A MANUAL FOR GENETIC ENGINEERING

Advanced Bacterial Genetics

GUILD

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PREFACE

In the last 5 years there has been a revolution in the way in which molecular genetics is practiced. The two major elements of the revolution were the development of methods for gene isolation and manipulation, collectively known as recombinant DNA, and the discovery and exploitation of translocatable drug-resistance elements (transposons). This manual contains experiments aimed at illustrating both of these new technologies in a way that can be easily assimilated by molecular biologists familiar with the essential principles of bacterial genetics. In a sense, this manual is a continuation (as opposed to a replacement) of the manual by J.H. Miller, Experiments in Molecular Genetics, published just 8 years ago. The basic manipulations are assumed to be known here; none of the experiments found in the earlier manual are repeated here.

One of the main problems faced by scientists wishing to apply modern genetic methods (especially molecular cloning) to their research is the large body of basic information and techniques in bacterial and phage genetics required. We hope that this manual (and courses at universities where this manual is used) can make this technology more accessible.

The basic framework of the manual was developed for a 3-week Advanced Bacterial Genetics course, which we have taught for the last 4 years at Cold Spring Harbor Laboratory. We can envision several ways in which the manual can be used elsewhere. First, from the number of requests for it in unpublished form, we expect it to be used in research laboratories as a source of experimental protocols. Second, we hope it will be useful in teaching an advanced laboratory course at the graduate level. We imagine (and recommend) that such graduate-level courses can be organized by picking some experiments from Experiments in Molecular Genetics and some from this manual.

One of the unusual features of this manual is the use of Salmonella typhimurium. Partly this reflects the interests and expertise of the authors. However, it has turned out to be a great advantage in teaching as well. The principles of Salmonella and Escherichia coli genetics are the same, yet the important manipulations involving transposons (especially those based on transduction) are easier and quicker in Salmonella. More important, the cloning of Salmonella genes in E. coli is a reasonable model for all kinds of molecular cloning: Genes can be expected to function (as do some yeast genes), but homology is sufficiently limited so that recombination between the cloned genes and the host is not a problem. Furthermore, the experiments illustrate nicely the relationship between in vitro genetic manipulations on a cloned gene in E. coli and the genetic analysis in vivo of the structure and function of that same gene in the organism from which it came. The advantages of studying organisms that are themselves manipulable genetically seem to us to have been, if anything, magnified by the development of recombinant DNA methods.

We are indebted to Peter Wensink and Jeffrey Miller, who taught early versions of this course with us at Cold Spring Harbor Laboratory. The course (and its contents) would never have existed without the help of the many assistants who labored over it and in it every summer. They are:

John Carlson, Forrest Chumley, Peter Gergen, Mark Johnston, Douglas Koshland, Steven Lam, Barbara Meyer, Paul Riggs, Mark Rose, Tom St. John, Stewart Scherer, Molly Schmid, Dan Stinchcomb, and Fred Winston.

The staff at Cold Spring Harbor Laboratory was enthusiastic and helpful, especially in the many moments of crisis. Ray Gesteland and, later, Jim Hicks, merit particular thanks. Ann Bushnell was invaluable in her support of our efforts to teach electron microscopy. Ann Strathern merits particular thanks for organizing the Strain Kit. Nancy Ford and her staff were remarkably tolerant and helpful throughout the preparation of the manuscript. Nadine Dumser and Marie Moschitta deserve special mention for their efforts in editing and preparing the final copy reproduced here.

We thank Alexander Kohn for permission to reproduce some of his cartoons, which we have interspersed at random throughout the manual. These inventive sketches, decorating the walls of Davenport Laboratory, have been a source of amusement over the years for both students and instructors alike. The captions were graciously supplied by Waclaw Szybalski.

We would also like to acknowledge the kindness of the following companies who provided free materials for use by students in the course: Bethesda Research Laboratories, Inc., Rockville, Maryland; Boehringer-Mannheim Biochemicals, Indianapolis, Indiana; New England Biolabs, Beverly, Massachusetts; New England Nuclear, Boston, Massachusetts; and Schleicher and Schuell, Keene, New Hampshire.

Finally, we are indebted to Jim Watson for his support of bacterial genetics as a science of continuing importance in modern molecular biology.

R.W. Davis
D. Botstein
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INTRODUCTION

Organization and Content

This manual is divided into three sections: Experiments, Procedures, and Appendixes. The thirteen interrelated experiments describe, in general terms, the design, rationale, and method employed to accomplish a particular end. Detailed protocols are provided as procedures, several of which may be involved in each experiment. The appendixes contain relevant information drawn upon in the other two sections. The experiments and procedures also contain discussion sections, which provide explanations and rationales, as well as some suggestions for further experiments and controls.

The experiments were in general designed to illuminate and to depend upon each other. For example, Experiment 7 constructs a genetic deletion map that is correlated to a physical map in Experiment 10 by using materials generated in Experiments 8, 9, and 12. The experiments were not designed to be done in any obligatory sequence and were carried out essentially simultaneously in the 3-week course given at Cold Spring Harbor Laboratory.

The procedures are intended to serve the experiments, but they can also stand alone. The particular methods can be used in many diverse endeavors, and the procedures were written to be as independent of each other as possible. The physical format is designed to facilitate use of the manual at the bench: Operational steps are segregated from the rationales, which are in the discussion sections. We hope that the procedures will be useful by themselves in the research laboratory.

The manual also contains a strain list that is keyed to the experiments and procedures. In some cases (e.g., cloning into λ vectors), many alternatives are given, only one of which is needed. The phage and bacterial strains are available as a kit and can be purchased from Cold Spring Harbor Laboratory.

Safety and Guidelines

Most experiments in this manual use Salmonella typhimurium. After many years of cultivation in the laboratory, S. typhimurium strain LT2 (from which all the strains used here are derived) no longer seems to be an active pathogen for man, although it is descended from strains that undoubtedly were capable of causing diarrhea. In the many years of using S. typhimurium LT2 in the laboratory, there have been no published cases of human infection, although some cases of human infection by similar strains (e.g., LT7 or 1559) have been reported. Strain LT2 is attenuated also with respect to mice, although adequate doses will still kill mice.

Therefore, all experiments should be carried out using prudent bacteriological practices. Cultures, and glassware that has been in contact with cultures, should be sterilized before washing, and waste (petri dishes) should either be sterilized before disposal or incinerated. Pi-

petting by mouth should be discouraged, although we tolerate pipetting of dilute suspensions of bacteria (and phage) by mouth.

Some of the experiments in this manual involve recombinant DNA experiments. However, these are exempted from the National Institutes of Health Recombinant DNA Research Guidelines, as S. typhimurium and E. coli are known normally to form chromosomal recombinants. Nevertheless, we exercise the same prudence with the recombinants as with Salmonella itself.

The experiments described here, when used with DNA from organisms other than Salmonella, may come under the NIH Guidelines, and therefore it is necessary to consult these Guidelines before undertaking such experiments. Since all the strains we use as hosts for recombinant DNA are derivatives of E. coli K12, virtually all experiments that are not forbidden will require at most P1-level physical containment.

Strain and Mutation Nomenclature

A uniform system of nomenclature is used for both E. coli and Salmonella genetic stocks. This system was originally described by Demerec et al. (1966). New conventions have been added to the system to accommodate the use of insertion elements. These modifications are described by Campbell et al. (1976) and Chumley et al. (1979). A summary of mutation types and their designations and map positions can be found in Bachman and Low (1980) for E. coli and in Sanderson and Hartman (1978) for S. typhimurium.

The Basic System: Each bacterial strain, regardless of the number of mutations carried, is assigned a stock number. This number refers to that particular isolate. In practice, strain numbers usually consist of two capital letters (designating the laboratory) and a serial numbering of the strains in the collection of that laboratory. For example, strain TR248 carries mutations hisC527 and cysA1349 and is in the collection of John Roth. New strain collections should use two-letter designations that have not been used previously.

Each mutant locus is given a three-letter designation (lower case, italicized), generally chosen on the basis of its gross phenotype or isolation method. (For example, mutations affecting histidine biosynthesis are designated his.) Individual genes at this locus or affecting this process are distinguished by an italicized capital letter following the three-letter designation. (For example, the hisC gene encodes a transaminase involved in histidine biosynthesis.)

Individual mutations are assigned numbers serially. A separate series is used for each three-letter locus designation. (For example, hisC527 refers to a particular his mutation; it affects the hisC gene. No other his mutation, regardless of the gene affected, will be assigned the number 527.)

Frequently, it is necessary to refer to the phenotype of a strain and to distinguish this from genotypic descriptions. Abbreviated phenotype

designations are not italicized and start with capital letters. (For example, strain TR251 [hisC527 cysA1349 supD] is a $Cys^+ His^+$ strain [phenotype] since the supD suppressor mutation corrects the auxotrophy of both the cysA and the hisC mutations.)

Use of transposable elements has necessitated additional conventions. Insertion mutations are given three-letter designations, gene designations, and allele numbers as described above. In addition, they receive a designation that describes the material inserted. This latter designation follows a double colon. (For example, hisC8691::Tn10 designates a particular insertion of the transposable drug-resistance element Tn10 within the hisC gene; the mutation was assigned the his allele number 8691.) In designating transposable elements, two capital letters designate the type of element. IS refers to simple insertion sequences that include no genes other than those involved in transposition. Tn refers to more complex transposons that include added genes such as those conferring drug resistance.

Many Tn10 insertions that are not known to be mutations of any particular gene have been isolated. Most are isolated as insertions near a gene of interest (see Experiment 2). A suggestion of Hong and Ames (1971) has been modified to permit naming Tn10 insertions according to map position when the insertion is not within a particular gene. All such insertions are designated by a three-letter symbol starting with z; the second and third letters designate approximate map position in minutes. The second position designates 10-minute map segments numbered clockwise from minute 0 (a = 0–10; b = 10–20; c = 20–30, etc.); the third letter similarly designates minutes within any 10-minute segment. For example, a Tn10 insertion located near hisW, between minutes 47 and 48, is designated zeh-754::Tn10; a Tn10 insertion located near his at minute 44 is designated zee-2::Tn10. Allele numbers are assigned serially to such insertions regardless of the letters appearing in the second and third positions. Thus, if more refined mapping data warrant a new three-letter symbol, the numerical identity of the insertion mutation is not lost. This convention uses designations zaa-zjj. We have designated insertion mutations on extrachromosomal elements with zz, followed by a letter denoting the element used. Designation zzf denotes insertion mutations on an F' plasmid.

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Demerec, M., E.A. Adelberg, A.J. Clark, and P. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics 54:61.

Hong, J.-S. and B.N. Ames. 1971. Localized mutagenesis of any specific small region of the bacterial chromosome. Proc. Natl. Acad. Sci. 68:3158.

Sanderson, K.E. and P.E. Hartman. 1978. Linkage map of Salmonella typhimurium. Microbiol. Rev. 42:471.

STRAIN LIST

All strains are S. typhimurium unless stated otherwise.

Experiment 1

NK337 hisC527 leu-414 supE (P22 c2ts29 12amN11 13amH101 int-3 Tn10)
LT2

Experiment 2

TR5989 (purB12)
Pool of Tn10 transposition clones (10,000)
P22 (HT, int$^-$)

Experiment 3

TT1127 (hisC8667::Tn10, orientation B)
TT1151 (hisC8691::Tn10, orientation A)
TT513 (zee-2::Tn10, orientation A)

Experiment 4

TT5371 (zeh-754::Tn10); Tn10 90% cotransduced with hisW
P22 (HT, int$^-$) phage grown on TT5371 and mutagenized LT2 cells

Experiments 5 and 6

DB4383 (E. coli) sup^o gal-1 gal-2 Sm^R lac$^-$ Amp^R
DB6430 (E. coli) argEam Rif^R/Nal^R Δ(lac pro)
DB6431 = DB6430 metB$^-$ supD (su1$^+$, inserts serine)
DB6432 = DB6430 metB$^-$ supE (su2$^+$, inserts glutamine)
DB6433 = DB6430 metB$^-$ supF (su3$^+$, inserts tyrosine, also called tyrT)
DB6434 = DB6430 metB$^+$ supP (su6$^+$, inserts leucine)
DB6435 = DB6430 metB$^-$ supG (su5$^+$, inserts lysine)
DB6436 = DB6430 metB$^-$ supB (inserts glutamine)
DB6437 = DB6430 metB$^-$ supC (inserts tyrosine)
λamp = λcI857 b515 b519 intam29 Tn2
λcI857 b515 b519 nin5 intam29
BNN45 (see Experiment 8)
λcI857 ind$^+$

Experiment 7

his Point Mutants

TR5583 hisO9657 (hisG1102, hisT1504)
TR5601 hisO9675 (hisG1102, hisT1504)
TR5589 hisO9663 (hisG1102, hisT1504)
TR5611 hisO9685 (hisG1102, hisT1504)
TR2895 hisG6570 (hisO1242)
TR2765 hisG6434 (hisO1242)
TR5063 hisG8642
TR6004 hisG708
TR5105 hisG8663 (hisO1242)
TR6005 hisG575
TR6006 hisG3037
TR6007 hisG572
TR6008 hisG460
TR6009 hisG2779
TR6010 hisD497
TR6088 hisD2578
TR6012 hisD3018
TR6013 hisD3794
TR6014 hisD3009 (ara-9)
TR1626 hisD3052 (uvrB)
TR6015 hisD492
TR6016 hisD113
TR6017 hisD10
TR6089 hisD476
LT2 wild type

P22 (HT, int$^-$)

his Deletion Mutants

TR5557 his-9615 (hisG1102, hisT1504)
TR5991 his-2321
TR5558 his-9616 (hisG1102, hisT1504)
TR5561 his-9619 (hisG1102, hisT1504)
TR5992 his-203
TR5993 his-1380
TR3067 his-8443
TR3321 his-8475
TR3456 his-8495
TR3335 his-8473 (dhuA1)
TR3325 his-8477
TR3320 his-8479 (edd)
TR5994 his-4902
TR6003 his-2236 (hisO1242)
TR5995 his-646
TR5996 his-2228
TR2880 his-6555
TR5999 his-712
TR6000 his-152
TR6001 his-2630
TR6002 his-129
TR2864 his-6539 (hisO1[illegible]
TR5997 his-2225
TR5998 his-3050

All of the above his mutants require only histidine for [illegible] minimal medium; additional mutations (none auxotrophic) are [illegible] parentheses. Each strain should be single-colony-isolated and [illegible] its his requirement before use. As controls, include [illegible] on LT2 (his$^+$, wild type) and TR5998 (his-3050, a deletion that removes the entire operon). Also use TR5998 as a control [illegible]

useful control recipient is an unrelated auxotroph (such as purB64 or purF145 in Experiment 13); phage lysates grown on all donor strains should give recombinants with these auxotrophs.

Experiment 8

λgt7-ara6
λgt7-S. typhimurium EcoRI pool
λgt4-lac5
RD100 (E. coli) hisB463
BNN45 (E. coli) hsdR$^-$ hsdM$^+$ supE44 (su2$^+$) supF (su3$^+$) B1$^-$ met$^-$

Experiment 9

λgt5-lac5,pBR322
BNN45 (see Experiment 8)

Experiment 10

See Experiment 7
RD101 = E. coli HB101[pBR322-hisOGD]

Experiment 11

DB5681 (E. coli) thi-1 (B1$^-$) lop-8 (ligase overproducer) (λimm434 cI-ts Sam7)

Experiment 12

HB101 (E. coli) hsdR$^-$ hsdM$^-$ recA13 supE44 (su2$^+$) lacZ4 leuB6 proA2 thi-1 (B1$^-$) Sm^R
RD102 = HB101/λ (resistant to phage λ adsorption)
RD103 = HB101[pBR322].

Experiment 13

Strain		Map position (minute)
TR5654	strA1 thrA9	0
TR5655	strA1 leu-485	3
TR5656	strA1 proA36	7
TR5657	strA1 purE8	12
TR5658	strA1 pyrC7	22
TR5659	strA1 purB13	25
TR5660	strA1 pyrF146	33
TR5662	strA1 his-2236	44
TR5663	strA1 purF145	49
TR5661	strA1 aroC5	49.5
TR5664	strA1 cysA533	52
TR5665	strA1 cysC519	60
TR5666	strA1 serA13	63
TR5667	strA1 cysG439	72
TR5668	strA1 cysE396	79
TR5669	strA1 ilv-508	83
TR5670	strA1 metA53	90
TR5688	strA1 purA155	93
TR5671	strA1 pyrB64	98
TT627	pyrC7 strA1/F'$_{ts}$114 lac^{+} zzf-20::Tn10 (orientation A)	
TT628	pyrC7 strA1/F'$_{ts}$114 lac^{+} zzf-21::Tn10 (orientation B)	
TT629	pyrC7 strA1/F'$_{ts}$114 lac^{+} zzf-22::Tn10 (orientation A)	

Procedure 3 — P22 (HT, int^{-})

Procedure 5 — NK337 (see Experiment 1)
TR4368 his-644 (P22 sieA27)
P22-503 (c1-7 12amN114 13amH101)

TR248 cysA1349am hisC527am

TR251 cysA1349am hisC527am supD ($su1^+$)

Procedure 7 — See Experiments 5 and 6

Procedure 9

λcI857 b515 b519 nin5 intam29

λamp = λcI857 b515 b519 intam29 Tn2

λcI857 ind^+

Procedure 11

DB5681 (see Experiment 11)

DB5683 (E. coli) thi-1 ($B1^-$) lop-8 (λcI857 Sam7)

Procedure 14

For detailed description of structures, see Appendix 9.

λgt1-λB

λgt4-O

λgt5-lac5

λgt7-ara6

λgt7-lac5

λ607

λsep6-lac5,lac5

Charon 4

λ590

λ760

λgt30-Ec6

λgt40-O

Procedure 15

RD104 = E. coli C600 $hsdR^-$ $hsdM^+$

A: E. coli N205 $recA^-$ (λimm434 cI-ts b2 red3 Eam4 Sam7)

B: E. coli N205 $recA^-$ (λimm434 cI-ts b2 red3 Dam15 Sam7)

Procedure 16

SF8 (E. coli) $hsdR^-$ $hsdM^-$ recB recC lop-11 (ligase overproducer) supE44 ($su2^+$) gal-96 Sm^R leuB6 thi-1 ($B1^-$) thr^-

HB101 (see Experiment 12)

BNN45 (see Experiment 8)

Procedure 17	RD102 = HB101/λ (see Experiment 12)
Procedure 18	HB101 (see Experiment 12) RD102 = HB101/λ (see Experiment 12) BNN45 (see Experiment 8) SF8 (see Procedure 16)
Procedure 19	BNN45 (see Experiment 8) RD105 (E. coli) trpC9830
Procedure 29	E. coli 594 (λpolA cI857 nin5 Qam73 Sam7) E. coli E1150 (λWam Eam T4lig cI857 nin5 Sam100)

SECTION I

EXPERIMENTS

EXPERIMENT 1

ISOLATION OF AUXOTROPHIC Tn*10* INSERTIONS

Introduction

Transposable elements can be added to the bacterial chromosome at many points. If the insertion site is within a bacterial gene, the linear continuity of that gene is disrupted and the gene function lost. When a drug-resistance element inserts within the gene for a biosynthetic enzyme, the resulting mutant has a dual phenotype. A nutritional requirement (auxotrophy) is caused by loss of the biosynthetic function; drug resistance is caused by expression of the inserted genetic information. These two aspects of the phenotype are consequences of the same insertion event and are completely linked in subsequent standard genetic manipulations (Kleckner et al. 1975).

These insertions and auxotrophs have proved to be extremely useful for a wide variety of genetic manipulations of bacteria (Kleckner et al. 1977). For example, they provide a means for selectively adding any particular auxotrophic marker to a strain of interest. One introduces the mutation from a donor carrying the auxotrophic insertion mutation (e.g., *hisG*9436::Tn*10*; a Tet^R, His^- insertion mutant). Selection is made for inheritance of drug resistance (a positive selection). Every recombinant that inherits resistance (by standard recombination events) must of necessity also acquire the donor's auxotrophy. (Transposition is sufficiently rare compared to recombination that it does not present a problem.) More examples of the uses of these insertional auxotrophs are described by Kleckner et al. (1977), Chumley et al. (1979), Chumley and Roth (1980), and Schmid and Roth (1980).

The means by which these insertion mutants are obtained is noteworthy as it results in recovery of mutants with high frequency, although the cells are exposed to very

low levels of mutagenesis. Each mutant isolated has suffered one, and only one, mutational event. Thus, one avoids isolation of mutants with multiple lesions, a problem associated with high levels of chemical mutagenesis.

Rationale

In this experiment, the transposable element Tn*10* (tetracycline resistance) will be introduced into *Salmonella* by means of a specially constructed P22 phage (Chan et al. 1972; Kleckner et al. 1975). The phage carries Tn*10* and mutations that block phage replication (*12*$^-$), lysis (*13*$^-$), repression (*c2*ts), and integration (*int*$^-$). The phage mutations prevent the donor from conferring tetracycline resistance (Tet^R) on the recipient by any standard means (lysogeny, plasmid formation). Since the phage shares no homology with the recipient chromosome, the Tn*10* element cannot be inherited by standard homologous recombination events. Under these conditions, selection is made for tetracycline resistance. The recipient must acquire the Tn*10* element by nonstandard, illegitimate means, that is, by transposition from the P22 chromosome into the recipient chromosome. Each Tet^R transductant colony is the result of an independent single transposition event, and each transductant carries one copy of Tn*10* DNA at a particular site in its chromosome. Under the conditions used, such Tet^R transductants occur with a frequency of $1/10^5$ infected cells.

In the actual cross, the multiply mutant P22 phage carrying Tn*10* in its genome (P22 Tc*10*) (Chan et al. 1972) is mixed with cells and plated on rich medium containing tetracycline. Only recipient cells that inherit Tn*10* (by transposition) are able to form colonies. After these colonies have grown up, they are replica-printed to rich medium (LB [Luria-Bertani broth] + Tet) and to minimal medium (E + Tet). These replica plates are compared to identify auxotrophs. Phage used in the cross is prepared by induction of the lysogenic strain NK337 as described in

Procedure 5. A method for assaying the titer of this defective phage is also described.

Method

Transposition of Tn10

1. Mix P22 phage (lysate of NK337) and wild-type Salmonella (LT2) cells at a multiplicity of less than one. Incubate to permit phage adsorption. Spread the mixture on plates of LB + Tet (20 μg/ml) + EGTA (10 mM) medium. Plate sufficient cells to obtain about 100–200 colonies per plate (see Procedure 5). Prepare 15 plates per group. Incubate plates at 40°C until colonies appear (about 24–36 hr).

Finding Auxotrophs

2. Replica-print each transduction plate to LB + Tet (20 μg/ml) + EGTA medium and to E + Tet (10 μg/ml). Incubate at 37°C.

3. Score plates by comparing rich and minimal replica plates. When colonies are encountered that appear on rich medium but not on minimal medium, pick the colony from the rich plate and streak for single colonies on a Green + Tet plate (no EGTA). Incubate streak plates at 37°C.

Classification of Auxotrophs

4. Pick a light-colored (phage-sensitive) colony from the streak of each potential auxotroph. Transfer each colony to a distinct position on a grid plate (Green + Tet + EGTA). Incubate plates at 37°C.

5. Replica-print the grid plate(s) of potential auxotrophs to the series of 11 diagnostic pool media (see Appendix 2), to minimal medium, and to Green + Tet + EGTA plates. Incubate all plates at 37°C for 24–36 hours. Save the original master plates.

6. Score replica plates. Identify the probable requirement of each auxotroph. Using cells from the rich-medium replica plate (Green + Tet + EGTA), repurify auxotrophs by single-colony isolation. Then, from a single colony, start a 2-ml liquid culture of each auxotroph. Incubate with agitation at 37°C.

Checking Phage Sensitivity and Requirements of the Mutants

7. Cross-streak a culture of each potential auxotroph with P22 phage on a Green + Tet plate (no EGTA) to check phage sensitivity. Spread 0.1 ml of each culture on a minimal plate and add a few crystals of the required nutrient, which was identified using the diagnostic media.

8. Score the phage sensitivity tests and nutrient crystal tests. Each auxotroph that is phage-sensitive and responds to the identified nutrient is ready for preservation. Make a permanent stab culture and enter the strain in the log book.

Discussion

1. P22 phage requires calcium to inject. EGTA chelates calcium and prevents phage multiplication. In this experiment, recovery of phage-sensitive transductants is enhanced by preventing rounds of phage growth on the plate. Preadsorption is required to permit phage injection before cells contact the plate containing EGTA. The transduction mix is plated at 40°C because the phage used carries a c2ts mutation, and high temperature prevents establishment of repression.

2. The EGTA is used to prevent phage-sensitive cells from being infected by any viable phage present on the transduction plate.

3. Rich and minimal media can be compared by holding them one above the other against a light. Frequently, auxotrophs leave a very faint imprint on the minimal plate and grow up well on rich medium. Green indicator plates (see Appendix 1 for description) are frequently used in experiments that involve Salmonella phage P22 (Levine and Curtiss 1961). Bacterial colonies in which P22 is actively growing turn dark green. Uninfected cells (and stably lysogenic cells) form light-colored colonies. The medium is basically a rich broth containing a pH indicator and a high concentration of glucose. Plates with no EGTA are used for streaking to permit visualization of phage-infected colonies.

4. Avoid dark-colored infected colonies on the streak plate. Pick the well-isolated, light-colored colonies. A pattern to use in placing colonies on the grid plate is found in Appendix 11. To avoid cross-contamination of patches during printing, keep patches fairly small.

5. The compositions of the 11 diagnostic media are found in Appendix 2. Each nutrient is found in two of the media. For example, serine is in Medium 2 (contents listed vertically) and in Medium 10 (contents listed horizontally). Thus, a mutant that grows only on Media 2 and 10 is likely to be a serine-requiring auxotroph.

6. Each auxotroph should grow on two of the diagnostic media (see above). Exceptions are auxotrophs with multiple requirements, such as those requiring both isoleucine and valine (which grow only on Medium 7) and those requiring all aromatic amino acids (which grow only on Medium 8). Medium 11 contains a collection of nutrients not appearing in any of the other

media; most of these are vitamins. Mutants growing only on Medium 11 must be tested for utilization of each individual component. (Also see Appendix 2.)

7. A 0.1-ml pipette is dipped into a suspension of P22 (c2) (clear-plaque) phage (10^8/ml). The pipette is then streaked across the surface of a Green indicator plate and the moisture is allowed to soak in. After the phage streak is dry, bacterial cultures (8–10/plate) are streaked in one direction across the phage streak using a sterile wooden applicator stick or an inoculating loop. Plates are incubated at 37°C.

8. Methods of storing bacterial strains are described in Appendix 3, and nomenclature conventions for mutations and strains are described in the Introduction to this manual.

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EXPERIMENT 2

ISOLATION OF Tn*10* INSERTIONS NEAR PARTICULAR GENES

Introduction

It is frequently useful to have a genetic marker near a gene of interest. Such a marker provides a way of moving that gene selectively into different genetic backgrounds and is also useful in local mutagenesis (see Experiment 4). Drug-resistance elements can be used to provide such a selectable marker at almost any point in the chromosome. This experiment involves isolation of Tn*10* insertions near, but not within, a particular region of interest (the *purB* gene).

Rationale

A random array of Tn*10* transposition clones is generated exactly as described in Experiment 1. Approximately 2000–5000 of such clones are pooled, and general transducing phage are grown on this mixture of bacterial insertion mutants. This phage preparation is used to transduce a mutant in the region of interest. Selection is made to restore function to that mutant (in our case, to replace the *purB* region). Each transduced fragment is derived from a donor cell carrying a Tn*10* insertion at some point in its chromosome. There is a reasonable probability (approximately 0.01) that the transduced fragment carrying the donor *purB*$^+$ gene will be derived from a cell that has acquired a Tn*10* insertion very near the region of interest. For such fragments, the Tn*10* element may be coinherited with the selected region. This is tested by scoring all transductants for tetracycline resistance. In practice, from 0.01 to 0.001 of the transductants will prove to be tetracycline-resistant (Tet^R); most of these will carry a Tn*10* insertion near the region of interest. (Some may be double transductants that have inherited a Tn*10* element at an unlinked site.)

Method

Making a Pool of Tn10 Insertion Mutants

1. Do a transposition cross (as outlined in Experiment 1). Adjust phage and cell concentrations so as to get approximately 500 transductant colonies per plate. Prepare ten plates. Incubate at 40°C for 2 days.

2. Add a few milliliters of LB broth containing EGTA (10 mM) to each plate. Suspend colonies in this liquid using a glass spreader, and pool the suspensions. Mix well. Dilute the suspension to approximately 10^8 cells/ml in LB + EGTA + tetracycline (10 μg/ml). Incubate overnight at 37°C with agitation.

Preparing Transducing Phage on the Pool of Insertion Mutants

3. Wash the culture of pooled insertion mutants twice by centrifugation and resuspension in E medium. Use the washed suspension to inoculate 1.0 ml of LB broth for preparation of a P22 transducing lysate (see Appendix 3). Save the remainder of the washed pool of insertion mutants.

4. Mix the fresh overnight culture with 4.0 ml of P22 Broth (LB broth medium containing 5×10^6 pfu/ml of P22 phage; see Procedure 3). Incubate at 37°C, with agitation, 5–18 hours or until lysis occurs. Even if no lysis is noticed by 18 hours, proceed with the steps below. This method of phage growth is described in Procedure 3.

5. Centrifuge to remove cells and cell debris. Pour the supernatant into a tube with 0.25 ml of chloroform. Mix vigorously with Vortex mixer to sterilize lysate. Final lysate should contain 10^{10}–10^{11} pfu/ml.

Isolation of the Tn10 Insertion Mutant

6. Start a culture of TR5989 (purB12).

7. Do a transduction cross on E medium using 0.1 ml of the fresh overnight culture of TR5989 (purB12) and a range of volumes (0.01–0.1 ml) of P22 phage grown on the pool of Tn10 random insertion mutants. Prepare about ten plates. Incubate at 37°C until transductant colonies are large enough to replica-print (36–48 hr).

8. Replica-print the transduction plates onto E + EGTA and E + Tet + EGTA (10 μg/ml) plates. Incubate at 37°C until ready for scoring (24–48 hr).

9. Identify transductants that grow both on E and on E + Tet plates. Pick transductants from plates containing tetracycline and streak on Green + Tet indicator plates for single colonies. Pick and streak 10–20 of these transductants if available. Incubate streak plates at 37°C until colonies are large enough to score for color (18–24 hr).

10. From each streak, pick a well-isolated, light-colored colony and inoculate a small (1.0 ml) culture in LB medium.

11. Cross-streak each culture with P22 (c2) phage on a Green plate to check phage sensitivity.

Check the Linkage of Tn10 to Mutation purB12

12. Start a culture of strain TR5989.

13. Transduce TR5989 (purB12) to tetracycline resistance using phage grown on each of the possible Tn10 insertions near purB. Mix 0.01–0.1 ml of phage and 0.1 ml of fresh overnight culture of TR5989 on the surface

of an LB + Tet (20 μg/ml) plate. Incubate at 37°C for 24 hours.

14. Replica-print each transduction plate onto E + Tet + EGTA and onto E + Ade + Tet + EGTA media. Incubate replica plates at 37°C (24–36 hr).

15. Score percentage of Tet^R transductants that become simultaneously Ade^+. Make permanent cultures of those strains that show reasonably close linkage (more than 10% coinheritance of Ade^+ and Tet^R). Go to the plate on which the original phage-sensitive Tn10 strains were saved, remove cells of the strains whose Tn10 insertions show linkage to purB12, and make a permanent stab culture. Enter the strain in the log book with the percentage of cotransduction of its Tn10 insertion with the purB mutation.

Discussion

1. This can be done at the same time as the transposition in Experiment 1.

2. It is important to keep EGTA in all media during the suspension and growth of these pools, since some phage is still present and could grow if EGTA were left out. The plan is for phage-sensitive clones to appear and persist during the growth period. A pool of about 10,000 transposition clones prepared in this way is included in the strain kit. It should be grown in the presence of EGTA and stored at -70°C. When used for phage propagation, the pool should be treated as described below.

3. The washing steps are to remove EGTA in preparation for growth of P22 transducing phage on the pool of cells. E medium is used because it contains calcium, but any other calcium-containing buffer would probably

work as well. The washed cell suspension can be stored at low temperature for use at a later time as a source of mutants (see Appendix 3 for storage methods). The P22 strain used in this and other general transduction experiments carries two mutations: int$^-$ (which prevents integration and stable lysogeny [Smith and Levine 1967]) and HT (which causes the phage to package the host chromosome with higher frequency [Schmieger 1972]). These two mutations provide for high transduction frequencies and allow one to recover transductants that are not lysogenic for P22. The pool of insertion mutants can be frozen at this point for later use (see Appendix 3).

4. P22 Broth (see Procedure 3) is quite stable even at room temperature but is usually stored at 4°C. During phage growth, lysis can be determined as a clearing of the culture or from the appearance of threads of cell debris. Even when no visible signs of lysis are noted, this procedure usually yields usable phage preparations.

5. Following Vortex mixing with chloroform, it is best to allow suspensions to sit at room temperature for several hours or overnight to enhance cell killing by chloroform. Thereafter, suspensions (with chloroform) should be stored at 4°C.

6. (Step 7) A range of phage concentrations is used to compensate for variability in titer of donor lysate and variability in transducibility of the recipient strain.

7. (Step 8) It is important to maintain selective conditions (E medium) on these replica prints, since otherwise the original recipient strain TR5989 (purB12) could grow.

8. (Step 9) To maximize chances of obtaining a nonlysogenic, phage-sensitive transductant, it is best to purify transductants as soon as possible after they can be scored clearly. Several Tet^R colonies are purified, as some may prove to be double transductants and carry Tn*10* insertions unlinked to *purB*. Others may show only weak linkage to *purB*.

9. (Step 10) Colonies in which P22 is growing actively will be dark green. Nonlysogenic strains are light (pale green). Stable lysogens will be slightly darker than nonlysogenic strains, but the distinction is difficult to score reliably.

10. (Step 15) At this stage it may be useful to save two transductants: one carrying *purB*$^+$ and Tn*10* and another carrying the *purB*12 mutation and Tn*10*. Both can be valuable for use in strain construction. An isogenic pair of strains (*purB*12 and *purB*$^+$) can be isolated by saving each transductant type from this final linkage test; both strains will carry Tn*10* near the *purB* gene.

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EXPERIMENT 3

DELETION MUTATIONS GENERATED BY Tn*10*

Introduction

One property of insertion-sequence and transposable drug-resistance elements is their ability to catalyze the formation of deletions (and other rearrangements) (Kleckner et al. 1979; Ross et al. 1979). This property can be exploited to generate a series of deletions in a region of interest. Such deletions are useful for genetic mapping or merely as stable (nonrevertible) genetic markers for use in a variety of selections.

Rationale

Many of the Tn*10*-generated rearrangements lose the drug-resistance determinants. Thus, if one starts with a strain having a Tn*10* insertion in a region of interest and selects for loss of drug resistance, the population obtained will be greatly enriched for deletions (and inversions) near the original Tn*10* site.

This experiment is made easier by a selection method developed recently by Bochner et al. (1980); the method permits positive selection for tetracycline sensitivity. Using this method, one can easily obtain a population of spontaneous mutants that is then screened for the desired deletion type. The selection method is based on the thought (not a proven fact) that tetracycline may enter cells by a mechanism intended for iron transport; this transport system may be blocked by the inducible Tn*10* resistance determinant. Blockage of the iron-uptake system leaves cells unable to grow on low concentrations of iron. By adding to the medium compounds that sequester iron, the growth of fully induced Tet^R cells may be limited by iron supply.

The experiment described below involves seeking deletions that are generated by a Tn*10* insertion near the

his operon. In addition to deletions, inversions may be encountered that prevent hisD expression, either by disrupting the hisD coding sequence or by separating the hisD gene from its promoter.

Transductional crosses with donor phage grown on a series of point mutants will allow determination of whether a deletion has occurred and how much of the hisD gene is removed.

Method

Selection of Tetracycline-sensitive (Tet^S) Mutants

1. Start cultures of:
   TT1151 (hisC8691::Tn10, orientation A),
   TT1127 (hisC8667::Tn10, orientation B), or
   TT513 (zee-2::Tn10, orientation A).

2. Dilute culture 1:100 to approximately 2×10^7 cells/ml. Plate approximately 1×10^6 cells (0.05 ml) on a plate of Bochner medium. Prepare approximately five plates for each strain, varying the number of cells plated from 5×10^5 to 5×10^6. Incubate at 37°C for 24–36 hours.

3. Pick colonies from the selection plate and streak for single colonies on plates of Bochner medium.

Identifying hisD Mutants

4. From the single colonies, inoculate grid plates with about 200 colonies in a regular array. Incubate grid plates at 37°C overnight.

5. Replica-print grid plates onto five media: E medium, E + histidine, E + histidine + Tet, E + histidinol, and LB medium. Incubate at 37°C overnight.

6. Score for tetracycline sensitivity and for failure to

grow on histidinol (*hisD*$^-$). Pick and single-colony-isolate *hisD* Tet^S mutants on nutrient broth plates (no Tet). From TT513 also save strains that grow on E + histidinol, but not on minimal medium.

Characterize New hisD Mutants

7. Inoculate liquid cultures of new *his* mutants from single colonies.

8. Spread 0.1 ml of the mutants on an E + low-histidine plate. Let moisture soak in. Add droplets of mapping phage lysates grown on a series of *his* point mutants (see Experiment 7). Keep plates level. Incubate at 37°C.

9. Score mapping crosses.

10. Save any characterized deletion or inversion mutant by making a stab culture, and enter it in the log book.

Discussion

1. The Tn*10* insertion in strain TT513 is located outside the operon at the promoter end. Strains TT1151 and TT1127 carry Tn*10* insertions within the *hisC* gene, which is located immediately promoter-distal to the *hisD* gene.

2. The selection will only work if a small number of cells (10^6 or less) is added to the plate. The selection will not work in the presence of excessive iron or manganese ions. Therefore, care should be used in media preparation.

3. Single-colony isolation is done to remove parental cells in the background. These parental cells can grow on the media that will be used to check for auxotrophy.

4. (Step 5) Strains carrying the desired rearrangements will grow on E + histidine medium, but not on E + histidine + Tet or on E + histidinol medium. Most of these will be deletions, but some may be inversions. In strain TT513, an inversion with a breakpoint within the operon, but distal to hisD, should be a histidine auxotroph able to use histidinol as a histidine source (hisD$^+$).

5. (Steps 7 and 8) These deletion-mapping crosses should be done as described in Experiment 7.

6. (Steps 6 and 9) Interpretation of results: From TT513 (Tn10 outside promoter and operon), any deletion damaging the his promoter will be His$^-$ and unable to use histidinol even if the hisD gene remains intact. Inversions generated in TT513 will be his auxotrophs if the breakpoint falls within the operon. They will fail to use histidinol if the breakpoint is between the his promoter and the distal end of the hisD gene. They will use histidinol only if the breakpoint of the inversion lies promoter-distal to the hisD gene.

   From the hisC insertions, deletions extending toward the promoter (or removing it) will be unable to use histidinol. Inversions whose breakpoints are between the his promoter and the promoter-distal end of hisD will also fail to use histidinol.

   In mapping crosses, deletions will fail to recombine with a contiguous block of point mutants. Inversions will recombine with all point mutants, except those located very close to either breakpoint. Inversions will fail to show His$^+$ recombinants when crossed with deletions that cross either breakpoint. One can verify the above statements by drawing a few diagrams.

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EXPERIMENT 4

LOCAL MUTAGENESIS

Introduction

Once a gene of interest has been localized in the chromosome, it is frequently helpful to be able to generate a variety of new mutations near that gene. A method for doing this was suggested by Hong and Ames (1971); they mutagenized a suspension of the general transducing phage P22 and then used it in a transduction cross, selecting for incorporation of a donor gene near the region of interest. Each donor fragment incorporated introduces a segment of the surrounding chromosome that has been heavily mutagenized. Thus, each transductant has a high probability of acquiring a mutation in any gene closely linked to the selective marker. Since only this tiny portion of the chromosome has been mutagenized, the probability of multiple mutations is much lower than for standard mutagenesis methods. (However, multiple mutations within the region of interest may be encountered.) Use of drug-resistance elements has made this technique more widely applicable. By using tetracycline resistance as a selective marker, the chromosomal region near any Tn*10* insertion can be heavily mutagenized. Since this selective marker (e.g., tetracycline resistance) can be placed close to any gene of interest (Experiment 2), the technique can be applied to any region of the chromosome.

Rationale

In this experiment, P22 (HT, *int*$^-$) phage is grown on a strain carrying a Tn*10* insertion near the essential gene *hisW*, at minute 47 on the *Salmonella* map (Anton 1968; Brenchley and Ingraham 1973). Cold-sensitive lethal mutations have been found in this gene; these mutants are constitutive for the *his* operon when grown at the permissive temperature. The object is to isolate a heat-sensitive lethal mutation by local mutagenesis of this region.

The phage preparation should be mutagenized with hydroxylamine by the method described in Procedure 8. This mutagenized phage preparation will be used to transduce Tn*10* (tetracycline resistance) into a wild-type recipient (LT2). The transductants will be tested for possession of a temperature-sensitive lethal mutation or a nonlethal, constitutive *hisW* mutation, identifiable by its wrinkled-colony morphology on plates with 2% agarose (Murray and Hartman 1972).

Method

Mutagenesis of the hisW Region

1. Start a small (3-ml) culture of LT2 from a single colony. Incubate at 37°C with agitation.

2. On each of 10 E + Tet + 2% glucose plates, spread 0.1 ml of LT2 cells and 0.1 ml of phage that has been grown on strain TT5371 (*hisW*$^+$ *zeh*-754::Tn*10*) and has been heavily mutagenized with hydroxylamine (see Procedure 8). Incubate plates at 30°C until tiny (pinpoint) colonies are visible (12–18 hr). Then shift plates to 40°C and continue to incubate until the majority of the colonies are large.

Identification of Mutants

3. Screen plates for tiny colonies (potential temperature-sensitive mutants) and for wrinkled colonies (potential *hisW* constitutive mutants). Pick tiny colonies and streak for single colonies on Green + Tet + EGTA plates. Incubate plates at 30°C. Pick and streak wrinkled colonies on plates of E + Tet + 2% glucose (6–8 streaks per plate). Incubate at 30°C.

4. Replica-print the streaks of potential temperature-sensitive mutants onto three E + Tet (10 μg/ml) + EGTA plates. Incubate one plate at 30°C, one at 37°C, and one at 41°C.

Pick a light-colored, wrinkled colony from the streak of each potential his constitutive mutant and proceed to check for phage sensitivity and linkage of wrinkled phenotype to tetracycline resistance.

5. Score the effect of temperature on the replica prints and, if possible, pick from each streak two colonies that show a clear effect of temperature on growth. Restreak these colonies on a plate of Green + Tet medium. Incubate plates at 30°C.

Check Mutants for Phenotype and Phage Sensitivity

6. Pick a light-colored (hopefully phage-sensitive) colony from each streak and patch it to a master plate of Green + Tet + EGTA medium. Incubate master plate at 30°C.

7. Replica-print grids of potential temperature-sensitive mutants to E-medium plates at 25°C (room temperature), 30°C, 37°C, and 40°C.

8. Score growth patterns after 24 hours. Grow up culture of each independent, verified, temperature-sensitive mutant and each wrinkled-colony mutant in nutrient broth at 30°C.

9. Check phage sensitivity by cross-streaking. If time permits, check linkage of the new mutation to Tn10.

10. Make permanent cultures and enter into the log book all verified temperature-sensitive mutants and all wrinkled-colony mutants.

Discussion

1. (Step 2) The phage suspension should be mutagenized such that only 0.1% of the plaque-forming units survive. Since virtually all mutations present in the

population were induced in the free phage particle by the mutagen, each mutant transductant recovered following the cross can be assumed to have resulted from an independent mutagenic event. The hope in this experiment is that temperature-sensitive mutants will form colonies at 30°C and that the colonies will not grow larger following the shift to high temperature. Thus, they will be detected as tiny colonies following incubation of the plates at 40°C.

2. (Step 3) Wrinkled colonies are formed by all his constitutive mutants when grown on medium containing a high concentration of glucose (Murray and Hartman 1972).

3. (Step 4) Patch plates are replica-printed to medium containing EGTA to prevent phage growth and to enhance the probability of recovering phage-sensitive, nonlysogenic recombinants. Since Green + Tet + EGTA medium contains 1% glucose, it is suitable for detection of the wrinkled colonies formed by constitutive mutants. Printing the streak plates and scoring all the isolated clones allows one to detect temperature-sensitive mutants even if the original colony contained a substantial number of revertants.

4. (Step 5) The second single-colony isolation is to free the strains of phage. This is done on Green + Tet plates without EGTA so that phage-sensitive colonies can be visualized.

5. (Step 7) Checks of temperature sensitivity are performed on minimal medium, since this allows clearer scoring. This may not be true for all mutants, and one might check sensitivity on both rich and poor media. It should be noted that this selection could

also yield temperature-sensitive mutants of the Tn*10* resistance determinant.

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EXPERIMENT 5

ISOLATION AND CHARACTERIZATION OF POINT MUTANTS OF PHAGE λ USING HYDROXYLAMINE

Introduction

Often it is useful to have phage mutations in a particular vector or clone without doing crosses that would introduce unwanted foreign information (such as restriction sites). Alternatively, it may often be desirable to isolate mutations in a cloned foreign DNA. The procedure is the same; all that is required is a scorable phenotype due to the function of the inserted DNA. Mutations in λ are useful as outside markers in crosses for mapping mutations in the cloned segment or as forcing markers to ensure recombination within the cloned segment.

Our model cloned segment is the TEM–β-lactamase present on λ as an insertion of the translocatable element Tn*2*.

Rationale

Procedure 8 is for hydroxylamine mutagenesis of phage in vitro. This method is very general for phage and DNA and has the virtue of being highly specific (G-to-A transitions) and very efficient. With temperate phage, it is easy to monitor the efficiency of mutagenesis by following the frequency of clear-plaque mutations among the surviving phage. The procedure will allow you to isolate amber mutants of λ and to characterize them by complementation with amber mutations in most of the known λ genes using a complementation spot test (Procedure 10). Complementation analysis will allow you to deduce the genetic map position of the mutations. The β-lactamase mutations can be used to help in mapping the deletion mutants of this model cloned segment (Experiment 6).

Method

1. Make overnight cultures of DB6430, DB6431, and DB-4383 (and any other suppressor strains you want to use; see strain list) in TYM broth for use in plating.

2. Prepare plating cultures; for this purpose, plating culture means cells growing exponentially at about 5×10^8 cells/ml. Titer λ*amp* stock by making serial dilutions, mixing 0.1 ml of dilution with 0.2 ml of plating culture, incubating at room temperature for 15 minutes, and plating on a λ or Red plate (Procedure 7) that can be incubated at 37°C or 34°C overnight.

3. Begin hydroxylamine procedure (Procedure 8). As you follow the procedure, titrate for survival and clear plaques at 34°C on λ plates. If you want to screen for amber mutations in λ genes, then also plate the mutagenized stocks on strain DB6431 (or another suppressor strain; see strain list), which carries a suppressor of amber mutations. For β-lactamase-negative mutations, plate on Red plates (Procedure 7).

4. Once the mutagenized stocks have been titered, plate several plates so as to get enough mutants.

Discussion

There are several ways to assess the β-lactamase phenotype in addition to Red plates. One is lysogenization of a host with the candidate phage, followed by a test of the resistance of the *lysogen* to ampicillin. This can be made semi-quantitative by using different concentrations of the drug. To do this, spot or streak a candidate phage on an ampicillin-sensitive (Amp^S) host (such as BNN45); in the particular case of λ*amp* (which carries an amber mutation in *int*), it should also carry *supE* or *supF*. Incubate plates at 34°C (λ*amp* has a temperature-sensitive repressor [*c*I857]) overnight. The next day, pick with a toothpick the center of the spots or plaques (where the lysogens are) into an LB + ampicillin plate, an LB plate, and (to keep the phage)

a λ plate seeded with any λ-sensitive host. The LB and LB + ampicillin plates have no added bacteria, since ampicillin resistance of lysogens is to be tested. The λ plate has added cells in soft agar, since phage is to be saved.

A second method is to spot phage suspensions directly onto a lawn of Amp^S cells (e.g., BNN45) on an LB + ampicillin plate. Only if the phage can lysogenize and confer ampicillin resistance, will any cells grow in the spot.

A third method is to use a chromogenic β-lactam (87/312 or nitrocefin) substrate. This can be applied directly to plaques or colonies.

EXPERIMENT 6

ISOLATION OF DELETION MUTANTS OF λamp

Introduction — One of the major advantages of λ as a cloning vehicle is the ease with which deletion mutations of a segment of foreign DNA in λ can be selected. The major structural and functional features of cloned DNA can be mapped quickly using deletion mutations; promoters, mRNAs, positions of structural gene information, etc., can easily be determined on a large number of strains with different end points.

Rationale — Phage is treated with a chelating agent (EDTA) that causes λ phage heads containing more than a specified amount of DNA (depending on conditions, particularly temperature) to be disrupted. It is possible to get conditions under which virtually the only survivors of EDTA treatment are deletion mutants. Since most λ vectors already have most of their nonessential DNA deleted, most of the deletions selected from a clone will be deletions involving the cloned foreign DNA.

Method — Carry out Procedure 9.

Discussion — The extent of deletions can be assessed in several ways. One way is to cross different deletions with each other. Another is to map them by DNA heteroduplex procedures. Still another is to test each of the deletions for its ability to recombine with point mutations and thereby construct a deletion map. You should, if time allows, try all of these methods. Detailed methods are given in Procedure 10. Since the emphasis is on the β-lactamase gene, you can use Red plates (Procedure 7) or lysogenization followed by resistance testing to score recombination to ampicillin resistance.

The lysogenization test is the most sensitive and can be done as follows. A λ plate seeded with BNN45 is prepared, and overlapping spots of the Amp^S deletions and/or point mutants are placed on the lawn. The plate is incubated overnight at 34°C. Cells from the center of each spot are transferred to LB + ampicillin and LB (as control) with a toothpick to test whether any ampicillin-resistant (Amp^R) lysogens were formed. Only if the Amp^S phage can recombine, will any such lysogens be formed.

EXPERIMENT 7

DELETION MAPPING

Introduction — Crosses involving deletion mutations provide the most reliable genetic method for ordering mutant sites in a genetic map. Restriction-fragment blot hybridizations provide data on whether deletions remove particular restriction sites in a DNA sequence. Together, the two techniques make possible a rough correlation between genetic and physical maps of a particular region. This correlation allows one to attribute the phenotypic defects characteristic of particular mutations to alteration of particular regions of the DNA sequence. The genetic mapping method and many of the mutants used were described by Hartman and co-workers (reviewed by Hartman et al. 1971). A more detailed map of hisG has been described (Hoppe et al. 1979).

Rationale — Each group should receive five his deletion mutants of Salmonella; each deletion affects the first third of the his operon (hisG, D, C). High-titer general transducing lysates grown on a series of his point mutations should also be provided. Each deletion mutant should be exposed to the entire array of donor phage by a spot test. The ability of each cross to yield His^+ recombinants will be scored (a "yes or no" test). This will permit rough mapping of deletion end points. The same deletions will be used in Experiment 10 for blot hybridizations. The combined results should allow positioning of restriction sites on the genetic map and allow estimation of the physical sizes of some deletions.

Method

1. From a single colony, start liquid cultures of each his deletion mutant and control deletion mutant purF145

(TR5663) or pyrB64 (TR5671). (These unrelated auxotrophs are among the strains used in Experiment 13.)

2. Spread 0.10 ml of each mutant on each of three plates (E + 0.005 mM histidine). Let fluid soak in completely. Using a collection of donor phage stocks in a Bertani box, drops of 25 different phage stocks can be transferred simultaneously to the surface of the cross plate. This is done with the aid of a frog (an array of 25 metal rods). Allow the plates to stand level until drops have soaked in. If such boxes are not available, drops of phage can be placed carefully on the recipient lawn with a pipette. Incubate plates at 37°C for 2 days. Include one plate with phage only (deposited on a plate with no recipient bacteria).

3. Score plates for presence of recombinants in spots where donor phage contacted the lawn of the deletion-mutant recipient. Use data to construct the best possible map of the mutations involved. If data do not permit an unambiguous map, some crosses can be repeated with higher resolution (0.1 ml of phage suspension and 0.1 ml of overnight culture on a full plate).

Discussion

1. (Step 2) Medium for deletion mapping includes a low level of histidine so that recipients can grow through several divisions. This enhances the number of transductants and thus the resolution of the mapping crosses. The pyrB (or purF) recipient is included as a positive control that demonstrates the efficacy of all phage lysates. Recipient deletion his-3050 is included as a negative control; this deletion removes all of the

his operon. These mapping crosses are especially sensitive due to the use of a mutant P22 as transduction phage. The phage carries a mutation (HT) that greatly increases the probability of incorporating host material into phage heads (Schmieger 1972). Approximately half of the phage particles in a lysate of this mutant phage contain host DNA (Susskind and Botstein 1978). In doing these crosses, it is helpful to preincubate the plates to be used overnight at 37°C. This will dry the plates so that moisture in the phage spots is more quickly absorbed, and it will help avoid confluence of the spots.

2. (Step 3) If spots on the control plate are free of colonies, then cross spots can be scored as positive (indicating recombination) even if as few as one colony arises. Normally, such a weak response would have to be confirmed by full-plate crosses (0.1 ml of phage suspension and 0.1 ml of a fully grown broth culture of cells spread on one selective plate).

References

Hartman, P., Z. Hartman, R. Stahl, and B.N. Ames. 1971. Classification and mapping of spontaneous and induced mutations in the histidine operon of Salmonella. Adv. Genet. 16:1.

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Schmieger, H. 1972. Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. 119:75.

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EXPERIMENT 8

SELECTION OF λgt-*his* CLONES BY COMPLEMENTATION

Introduction

Genes cloned in λ vectors can be selected from a pool of hybrids if these genes are expressed in *E. coli* and can complement a host-cell genetic defect. There are two basic ways in which this complementation testing can be conducted. The first is to form a lysogen with the hybrid phage. The lysogen is then tested for growth on selecting media. However, many λ vectors have been deleted for λ genes necessary to form lysogens. In these cases, a helper phage can be used to permit formation of double lysogens. The second method for selecting particular λ hybrids is lytic complementation. Normally, λ phage is unable to multiply in a host that is unable to grow due to some genetic defect. However, if the infecting phage carries into the cell the genes that complement the host deficiency, the cell can both grow and support multiplication of the phage. The cell is ultimately killed by the infecting phage and a plaque is produced.

Rationale

A phage λ hybrid pool has been constructed by inserting random *Eco*RI-generated fragments from *S. typhimurium* DNA into a λgt7 vector. The original hybrid pool was composed of 250,000 independently generated hybrids. Phage λ does not infect *Salmonella*, but *Salmonella* genes are expressed in *E. coli*. The complementation test can be done by infecting *E. coli* His^- cells. For the lysogenic selection, double lysogens will be formed using a helper phage that is called λgt4-*lac*5. This phage has all the essential genes for forming a lysogen; it also carries the *E. coli* β-galactosidase gene so that it can be distinguished from all the hybrids. The integration helper also carries the *c*I857 temperature-

sensitive mutation in the λ repressor gene. This will allow temperature induction of the lysogen for recovery of the hybrid phage. The lysogenic selection is then conducted by infecting the His^- cell with the hybrid pool and the integration helper and plating on minimal plates without histidine. Colonies that arise on these plates could be cells containing a hybrid phage that complements the defect; they could contain a hybrid phage that supresses the His^- lesion, or they could simply be revertants. Since a large amount of phage is used, colonies arising by any of the above mechanisms are likely to contain an integration helper and one or more hybrid phage. To distinguish between complementation, supression, or reversion, phage is recovered from colonies growing on the minimal plates. This is done by growing the culture for a short period of time at 42°C, which causes temperature induction. Hybrid phage in these cells can be distinguished from the integration helper by plating in the presence of Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), a chromogenic substrate of β-galactosidase. Hybrids form clear plaques and integration helper forms blue plaques. The hybrid phage can then be retested for complementation of the original mutant as well as other mutants.

The lytic complementation is much simpler. His^- cells are infected with the hybrid pool and plated on minimal plates without histidine. Phage complementing His^- cells will form plaques; however, the cells cannot grow to form a lawn. As a result, if plated in a normal fashion, plaques (clear spots) will be produced on a clear plate. Therefore, it is important to put down onto the plates a large amount of cells to cause a slight turbidity. When this is done, clear spots can be seen faintly. Since λ is actively transcribed during lytic infection, it is likely that the cloned sequences in the central region of λ are also transcribed from a λ promoter. Therefore, if the inserted gene does

not have its own promoter, it may be expressed during a lytic infection. As a result, phage found by lytic infection may fail to complement as a double lysogen. Since the plaque has been produced by several sequential infections, cellular reversion generally will not lead to the formation of plaques with noncomplementing phage.

Method

1. Start from a single colony and grow 40 ml of E. coli RD100 (hisB463) in minimal medium plus histidine and maltose (not glucose). After growth to near saturation, sediment and resuspend in 1/10 volume of 10 mM $MgSO_4$ to give 10^{10} cells/ml. Also, grow 40 ml of BNN45 on TYM broth for general λ plating (see Procedure 1).

Lysogenic Selection

2. Plate λ hybrid pool on E. coli His^- cell line following lysogenic selection. Use λgt4-lac5 as integration helper. Plate for each selection:

   10^8 λ hybrids and 2×10^9 λgt4-lac5 on one minimal plate minus histidine.

   10^7 λ hybrids and 2×10^9 λgt4-lac5 on one minimal plate minus histidine.

   10^6 λ hybrids and 2×10^9 λgt4-lac5 on one minimal plate minus histidine.

   Use 5×10^8 cells per plate. Grow at 32°C since the integration helper carries the cI857 temperature-sensitive mutation. Also plate individually the λ hybrids, the λgt4-lac5, and the His^- cells to check for contamination or reversion.

Lytic Selection

3. Plate λ hybrid pool on E. coli His$^-$ cell line following lytic selection procedure. Pour plates without ethidium bromide in top agar. Use 2×10^6, 2×10^5, and 2×10^4 λ hybrid phage and 2×10^9 cells per plate. Be sure to use M9 minimal plate and M9 minimal top agar without histidine. Incubate at 37°C.

Check complementation experiment (lytic and lysogenic). Plaque-purify four putative lytic complementers on LB plates (nonselective medium). Pick four colonies from the lysogenic complementation and place in 1 ml of LB broth. Grow at 32°C until there is a slight turbidity (10^7–10^8 cells/ml). Grow for 2 hours at 42°C to induce lysogens. Streak out resulting culture on LB plates for single plaques. Use 20 µl of RD100 or BNN45 cells for lawn on LB plates. Add 40 µl of 40 mg/ml Xgal to soft agar for lysogenic complementer only to identify integration helper (λgt4-lac5).

Complementation Retesting

4. Retest putative complementers on M9 minimal plates. Pick plaques from LB plates and resuspend each in 100 µl of λ dilution medium (λ dil). Choose four nonblue phage from the lysogenic selection and four phage from the lytic selection. Streak all eight on one M9 minimal plate with His$^-$ cells following lytic selection and all eight on one M9 minimal plate with His$^-$ cells plus 2×10^9 integration helper phage. Use the remainder for a plate stock on LB agarose plates. Harvest plate stock after incubating for 6 hours at 37°C. Overlay with 5 ml of λ dil at 5°C overnight.

Harvest complementation plate stocks and store in a sealed test tube over a few drops of chloroform.

Check complementation retest.

Rapid λ DNA Isolation from Phage Stock (hybridizing and complementing)

5. Follow Procedure 11-II for rapid λ DNA isolation. Use stock from agarose plates.

Distribution of Restriction-fragment Lengths of λ Hybrid DNAs

6. Cleave 5 µl of each DNA with EcoRI and load onto a 0.7% agarose gel. Electrophorese for 6 hours at 50 V. Save the remaining rapid lysate phage stock for making large sets of plate stocks.

Plate Stocks

7. Make 20 plate stocks for each of three to six phage based on results from gel electrophoresis (above). Grow for 6 hours and overlay with 5 ml of λ dil at 5°C.

Harvest Plate Stocks

8. Follow Procedure 4 for λ phage isolation. Use a minimum of tubes; 20 plate stocks can be placed in two 45-ml centrifuge tubes.

CsCl Purification of Phage

9. Resuspend phage pellet in a total of 1 ml (0.5 ml for each of two tubes). Follow Procedure 4-IA for the down block gradient.

EXPERIMENT 9

PLAQUE HYBRIDIZATION

Introduction

A particular hybrid phage can be isolated by its DNA sequence homology with a radioactively labeled RNA or DNA. The procedure used here allows the screening of a very large number of hybrid phages.

Rationale

A large amount of phage and phage DNA is present in a λ plaque. Phage and DNA can be directly transferred to a solid support by dropping a dry nitrocellulose filter into the plate. Once denatured, the DNA is available for hybridization. A maximum of 25,000 plaques can be screened on a single plate. Filters are hybridized with DNA labeled with ^{32}P by nick translation. After the filters are washed, they are exposed to X-ray film, and dark spots mark positively hybridizing clones.

The major technical problem is orientating the X-ray film with the original plate. For this, we will use an orientating phage. This phage contains a section of DNA that will hybridize with the nick-translated DNA (pBR322) and it contains the β-galactosidase gene of *E. coli*. The incorporation of Xgal into the plates results in a blue plaque being produced for each orientating phage. This phage will also hybridize with a pBR322 DNA probe. This orientating phage can be put onto the plates in a random array or spotted into a specific array. Nonblue plaques in the vicinity of the hybridization are picked, plaque-purified, and retested by hybridization.

Method

1. Nick-translate about 0.5 μg of DNA (Procedure 22).

Plaque Plate for In Situ Hybridization

2. Each group should prepare eight plates containing 10^4

and 10^3 phages on LB plates with LB soft agar. Incubate at 37°C. Use 2-day-old plates. Use 20 µl of BNN45 plating cells for each plate. Incorporate 10–20 λgt5-lac5,pBR322 phage into the plate or spot with straight platinum wire from a stock of λgt5-lac5, pBR322 phage. Also, incorporate Xgal into the plate (40 µl of 40 mg/ml in dimethylsulfoxide added to soft agar).

Plaque Filter Hybridization

3. Use two plates with about 10^4 plaques and two plates with about 10^3 plaques. Take each group's best two plates with 1×10^3 to 3×10^3 plaques and two plates with about 10^4 plaques. Follow Procedure 21-II.

   Be sure to mark and orientate each filter. Place filters in a heat-sealable bag with denatured, nick-translated DNA as in Procedure 23, and seal the bag. Be sure to wear double gloves, safety glasses, and film badge.

Plaque Filter on Film

4. Cut hybridization bag along top of seal and remove hybridization mix. Cut open bag and remove filter. Wash filter as described in Procedure 23. Store probe in refrigerator for reuse. Be sure to wear double gloves, safety glasses, and film badge. Dry filter and wrap in plastic wrap so that the film holders and intensifying screens do not become contaminated. Expose filter to X-ray film (see Procedure 24).

Develop Plaque Filter Film

5. Line up original plaque plate and pick up to eight plaques in positive-plaque area with platinum wire. Streak out for single plaques by the overstreaking method using 1/8 of the plate. Pick up to four positives. Incubate at 37°C for at least 6 hours. Pick

single plaques into a grid and incubate at 37°C overnight.

Plaque Filter Hybridization II

6. Prepare a new plaque filter replica from the plaques streaked for single plaques and picked into a grid. After baking, place new filter replica into a new bag, add hybridization probe, and seal. Be sure to wear double gloves, safety glasses, and film badge. Start this procedure early in the day. Store plaque plate in refrigerator.

Plaque Filter on Film

7. After hybridization of plaque filter II for at least 6 hours, remove filter, wash, blot dry, wrap in plastic, and place on film. Use different film holder than that used for gel transfer sheet if necessary. Expose for about 6 hours.

Develop Film of Plaque Filter II and Make Agarose Plate Stocks

8. After approximately 6 hours, develop the film of plaque filter II. Identify positive plaques. Pick plaques and make plate stock. Use agarose plates and follow Procedure 11-II for rapid phage DNA isolation. Use one plate for each phage (one to six). After 6 hours of growth, overlay with 5 ml of λ dil and incubate at 5°C for 2–12 hours. If time permits, prepare DNA rapid lysates of λ stocks.

Distribution of Restriction Fragment Lengths of λ Hybrid DNAs

9. Cleave 5 µl of each DNA with EcoRI and HindIII and load onto a 0.7% agarose gel. Electrophorese for 6 hours at 50 V. Save the remaining rapid lysate phage stock for making a large set of plate stocks.

Plate Stocks

10. Make 20 plate stocks for each of three to six phage based on results from gel electrophoresis. Grow for 6 hours and overlay with 5 ml of λ dil at 5°C.

Harvest Plate Stocks

11. Follow Procedure 2 for λ phage isolation. Sign up for centrifuge. Use a minimum of tubes; 20 plate stocks can be placed in two 45-ml centrifuge tubes.

CsCl Purification of Phage

12. Resuspend phage pellet in a total of 1 ml (0.5 ml for each of two tubes). Follow Procedure 4-IA for the down block gradient.

13. Continue phage purification.

Discussion

Nick translation can be a rather long procedure and should be started as early as possible. Be sure to wear radiation badge, lab coat, safety glasses, and double gloves. Also, use hand monitor to check constantly for radioactive contamination (see Procedure 22). [^{32}P]dCTP or other [^{32}P]dNTP, DNase I, dNTP, and 10× NT reaction buffer, NT stop reaction, and TE should be provided.

EXPERIMENT 10

GEL HYBRIDIZATION

Introduction

The sizes of restriction fragments homologous to specific probe DNAs can be determined directly from wild-type S. typhimurium and a large number of deletion mutants. In fact, many of these deletions can be ordered and their end points determined approximately by these hybridization procedures.

Rationale

DNA is prepared from a number of Salmonella strains. The DNA is isolated directly from colonies by a rapid procedure. This isolated DNA is then cleaved with a variety of restriction endonucleases, electrophoresed on an agarose gel, and then transferred to nitrocellulose paper. DNA labeled with ^{32}P by nick translation is then hybridized to the nitrocellulose strips. After exposure to X-ray film and development, the sizes of restriction fragments homologous to the probe can be determined.

Method

Rapid DNA Isolation

1. Isolate the DNA directly from colonies of strains in Experiment 5. Small, 1-ml liquid cultures may also be used. Follow Procedure 12-II for isolation of DNA from colonies. Resuspend DNA in 50 µl of 10 mM Tris (pH 7.5), 10^{-4} M Na_2 EDTA, and 10 µg/ml of RNase.

Restriction Endonuclease Digestion

2. Digest 10 µl of the above DNA with EcoRI, 10 µl with HindIII, and 10 µl with EcoRI plus BamHI, or EcoRI plus HindIII, or HindIII plus BamHI.

Gel Electrophoresis

3. Load entire cleaved DNA sample in one slot of a 0.7% agarose gel. Use Tris-acetate buffer. Electrophorese overnight (20 V for 18 hr or 30 V for 12 hr).

Transfer DNA in gel to nitrocellulose using Procedure 21-I. Transfer overnight.

Hybridization to Restriction Spectrum

4. Remove nitrocellulose sheet from gel, and wash and bake filter. Place into a heat-sealable bag and add probe as described in Procedure 23.

After at least 24 hours of hybridization, remove gel-transfer nitrocellulose sheet from bag, wash, blot dry, wrap in plastic, and place on film.

Develop gel-transfer film. Record total exposure time. Place on new film if underexposed.

EXPERIMENT 11

ELECTRON MICROSCOPY OF DNA

Introduction

Contrary to common belief, electron microscopy of DNA is a simple, fast, and reliable technique. Since individual molecules are examined and all molecules can be visualized, it complements gel electrophoresis techniques very well. In gel electrophoresis, only populations of molecules having a homogeneous length are seen. In addition to general visualization of DNA, heteroduplexes will be examined to map the positions of nonhomology regions.

Rationale

DNA will first be visualized using the aqueous technique and uranyl acetate staining. This is a very fast technique and can really aid experiments when it is required that the size or structure topology of a DNA sample be determined in a qualitative way. The second part of this experiment will focus on the construction of heteroduplexes and visualization of these heteroduplexes by the formamide mounting procedure. Initially, phage should be provided for carrying out the heteroduplex analysis. Later, deletions produced in the course can be mapped.

Method

1. Electron microscope demonstration and checkout.

2. Demonstration of aqueous DNA preparation for electron microscopy. DNA should be provided. Follow Procedure 27.

3. Demonstration of formamide DNA preparation for electron microscopy. Phage stocks should be provided for heteroduplex analysis. Follow Procedure 28.

4. Take photographs of heteroduplexes from your best grid.

5. Electron microscope heteroduplex analysis of interesting phage.

EXPERIMENT 12

SUBCLONING FROM λ TO A PLASMID VECTOR

Introduction — DNA cloned in λ will be transferred to a plasmid vector. Since the mass of the plasmid vector is smaller than that of λ, many restriction mapping procedures and sequencing procedures are more readily carried out.

Rationale — A plasmid vector, pBR322, will be used for the subcloning. This vector is Amp^R Tet^R. The plasmid DNA will be extracted and sedimented to equilibrium in cesium chloride with ethidium bromide. For the subcloning, any one of a number of enzymes can be used, with the particular enzyme to be determined prior to the subcloning. Most likely, however, EcoRI will be used, since this is the enzyme used to generate the hybrids. The subcloning can be facilitated by using two restriction enzymes (i.e., both vector and fragment to be subcloned are digested with two different enzymes, such as EcoRI and SalI.

Method

1. Streak out RD103 = HB101[pBR322] for single colonies.

   Test RD103 for tetracycline and ampicillin resistance. Save correct culture.

   Start 100-ml overnight culture of RD103.

pBR322 DNA Isolation

2. Follow Procedure 12-I for plasmid DNA isolation using 100 ml of HB101 with pBR322. Use volumes for one type 50 Ti rotor tube.

   Remove pBR322 DNA from gradient and extract the ethidium bromide from the DNA. Ethanol-precipitate the DNA or dialyze against 0.1 M NaCl, 0.05 M Tris (pH 7.5), and 0.1 mM Na_2 EDTA.

   Grow overnight cultures of RD102 = HB101/λ.

Extract λ DNA

3. Follow Procedure 11-I for λ DNA extraction, using about half of the phage stock.

Transfer DNA fragment(s) in λ to pBR322. Cleave with EcoRI or with combinations of restriction endonucleases. Use a mass ratio of 20–50 parts of λ hybrid DNA to 1 part of pBR322 DNA. Covalently join with T4 DNA ligase. Transfect into RD102 using either tetracycline or ampicillin selection.

EXPERIMENT 13

Tn*10*-DIRECTED INSERTION OF F'$_{ts}$ *lac*$^{+}$

Introduction

Transposable elements can act as a region of homology to permit a variety of genetic manipulations. Among these are formations of deletions, duplications, transpositions and, presumably, inversions of chromosome segments. The object of this experiment is to try one such operation. Tn*10* will be used as a region of homology between an F' episome and the bacterial chromosome. Recombination between such Tn*10* elements can cause insertion of the F' episome into the chromosome, thereby generating an Hfr. By determining the origin and direction of transfer of this Hfr, one has a useful means for learning the map position and orientation of a Tn*10* element in the bacterial chromosome (Chumley et al. 1979).

Rationale

Each group will use two random, uncharacterized Tn*10* insertions in the *Salmonella* chromosome (derived from Experiment 1). It should be possible to determine the map position and orientation of the Tn*10* element. The basic idea is to insert an F'$_{ts}$ Tn*10* *lac*$^{+}$ plasmid into the chromosomal Tn*10* element. The resulting Hfr can be mated with a variety of recipients to determine its point of origin and direction of transfer. This fixes the location and orientation of the Tn*10* sequences.

Method

1. Start an LB culture of the strains carrying a chromosomal Tn*10* insertion. Also start cultures (at 30°C) of strains carrying the F'$_{ts}$ Tn*10* *lac*$^{+}$ episomes (TT627, TT628, TT629).

2. Transfer the F'$_{ts}$ episomes into each of the Tn*10*

mutants (random insertions). This can be done by placing drops of the cultures on a selective plate (NCE + lactose). One spot on the plate receives a drop of donor; another spot receives a drop of recipient; a third spot receives a drop of both cultures. After moisture has soaked into the plate, each of these spots is streaked out independently on a portion of the plate. This assures that the mating mixture will yield single colonies of exconjugants and not just a confluent area of growth. Incubate plates at 30°C for 36–48 hours.

3. Pick several large colonies from the mating spot. (No colonies should appear in the control spot.) These are recipients that have received the F'_{ts} lac^+ plasmid. Streak each colony on both of two NCE minimal *lac* plates. Incubate one plate at 40°C and the other at 30°C.

4. After 48 hours, the 30°C streak should have grown up; the 40°C streak should show only a few large colonies. These are the putative Hfrs; they are lac^+ at a temperature (40°C) that prevents autonomous replication of the F'_{ts} lac^+ episome. Presumably, the F' lac^+ genes have escaped the temperature sensitivity by inserting into the chromosome. Pick several putative Hfrs and grow up in liquid selective medium (NCE + 0.5% lactose) at 40°C. Also start LB cultures (37°C) of a set of recipients to be used for mapping. These are single auxotrophs each carrying a streptomycin-resistance mutation. Since quite a few strains are involved, a cooperative venture can be set up and one set of recipient cultures can be shared with several other groups. Recipient control plates can also be done in common to save plates.

5. Do the plate matings to map Hfr origins. For the first

crosses, donor and recipient strains (0.1 ml of each) are plated directly on selective medium (E + streptomycin). Incubate plates at 37°C.

6. Score the number of recombinants referring to the map positions of the recipient markers. It is possible to map the orientation and approximate location of the origin of Hfr transfer. To refine the mapping, try the closest markers, again using dilutions of the donor. Further refinement would require time-of-entry mapping or three factor crosses.

Discussion

1. The F'_{ts} episomes carry a mutation that prevents their replication at 40°C. The episomes also carry a wild-type *lac*$^+$ operon and a Tn*10* insertion of known orientation. The episomes in strains TT627 and TT629 carry Tn*10* in the same orientation (A). Strain TT628 carries Tn*10* in the opposite orientation (B) vis à vis the direction of transfer.

2. *Salmonella* is naturally Lac$^-$ but can become Lac$^+$ by receiving an *E. coli* F' *lac*$^+$ episome. These plates must remain at 30°C to permit transfer and replication of the mutant F' episome.

3. A class of tiny colonies may also appear in the streaks from the mating spots. These are Lac$^-$ recipient colonies growing on nutrients added in the drop or by cross-feeding by the large Lac$^+$ colonies. Pick only large colonies for this experiment.

4. Hfr strains are grown under selective conditions to avoid excision of the F' *lac*$^+$. Rapid isolation and use of these Hfrs is important. If the Hfrs are handled extensively (i.e., repurified or stored) before use, they seem to undergo rearrangements that complicate interpretation of results. If one selects the Hfr and

uses it promptly, Tn10 insertions can be orientated unambiguously.

Reference Chumley, F.G., R. Menzel, and J.R. Roth. 1979. Hfr formation directed by Tn10. Genetics 91:639.

SECTION II

PROCEDURES

PROCEDURE 1

PLAQUE PURIFICATION OF PHAGE

I. Host Cells

1. Grow overnight culture of a λ-sensitive strain (e.g., BNN45) on LB or TYM broth.

2. For λ plating, sediment host cells (8000 rpm for 5 min), resuspend in 1/2 volume of 0.01 M $MgSO_4$, and store at 4°C. For P22, dilute and grow cells to exponential phase, chill, and store at 4°C in growth medium.

II. A. Understreaking

1. Streak phage on a λ or LB agar plate with a loop or fine wire.

2. Add 20–100 µl of plating culture to 2.5 ml of soft agar at 47°C.

3. Pour onto plate but not directly onto location where phage streak was started.

4. When agar has hardened, invert plates and incubate at appropriate temperature. Note that λ will not grow below 30°C.

B. Overstreaking

1. Add 20–100 µl of plating culture to 2.5 ml of soft agar at 47°C.

2. Pour onto λ or LB plate and allow to solidify completely.

3. With a very fine platinum wire or sterile strip of newspaper (1-1/2 × 1/8-inch), carefully streak the phage over the surface of the agar.

4. Incubate at 30–42°C.

III. Picking Plaque

1. Pick plaque (plug) with a sterile glass capillary or micropipette (10–50 µl).

2. Suspend in 20 µl to 1 ml of λ dil medium or plating cells, as appropriate. For storage, use 1 ml of λ dil and add 1 drop of chloroform to kill bacteria.

IV. Titering Phage

1. Make serial dilutions of phage in λ dil (10 mM Tris [pH 7.5] and 10 mM $MgSO_4$).

2. Mix 1–200 µl of diluted phage containing an estimated 100 phage with 20–100 µl of plating cells.

3. Incubate at 37°C or 25°C for 15 minutes to preadsorb phage to cells. This step is not necessary for P22.

4. Mix with 2.5 ml of soft agar at 47°C.

5. Pour onto λ or LB plate.

6. Incubate at 32–42°C.

Discussion

I. The plating cells can be from any strain that will give good plaques. Many E. coli strains are resistant to λ infection (designated /λ). Most amber mutations in λ can be suppressed by supE ($su2^+$), which is present in strain C600. The λSam7 mutation in many λ strains is suppressed by supF ($su3^+$), which is present in the host strain BNN45 in addition to supE. The bacterial protein to which λ adsorbs is encoded in the mal operon, which is induced by maltose. Thus, cells grown on maltose (TYM) adsorb λ very efficiently. However, the plaque plates should not contain maltose, because readsorption on the plate reduces final phage yield. The saturated culture can be stored in $MgSO_4$, or, if used immediately, the culture can be used directly. The $MgSO_4$ culture can be stored at 5°C for up to 2 weeks, but higher plating efficiencies and more uniform plaque morphology is achieved with cells no older than 1 day. P22 plating is best on exponential cultures of Salmonella stored in the cold no longer than 36 hours.

II. A. Streak the phage in the same manner as a bacterial culture. Use either a λ plate or an LB plate. Larger plaques are obtained on λ plates. The use of fewer cells will also give larger plaques. Since the phage is not preadsorbed, the infection that initiates the plaque will occur over a long time interval. As a result, the plaques may be variable in size. In this case, variable plaque size does not necessarily indicate heterogeneity in genotype of the phage. This method works well for those with little experience but it has the disadvantage that only one phage can be purified per plate.

B. This method is more difficult than A, but has the advantage that several phage can be streaked on one plate. Streaking is easier if the top agar is hard. Cooling the plate will help set the agar.

III. Generally, plaques can be seen after about 6 hours. The plaque will stop increasing in size as soon as the cells become stationary. However, the phage continue to diffuse in the top agar at about 1 cm/day. Therefore, to prevent contamination from phage in adjacent plaques, the most isolated plaque should be picked as soon as all plaques are clearly visible. Since one cannot be assured that the plaque is completely free of contamination, a second plaque purification generally is recommended.

IV. One-hundred-fold dilution is generally made as 0.1 ml into 9.9 ml of λ dil or 10 μl into 0.99 ml of λ dil. One-thousand-fold dilution can be made with 1 μl into 1 ml of λ dil. If the stock is several months old, the titer may have dropped by more than a factor of 10. Therefore, it is advisable to also plate some lower dilutions when assaying old stocks. A good practice when marking plates is to record the raw data concerning plated dilution. For example, record the dilution exponent from the final dilution and the volume plated. If one plates 0.05 ml from a 10^{-7} dilution, record 7/0.05.

PROCEDURE 2

PREPARATION OF PHAGE STOCKS

I. Host Cells (see Procedure 1)

II. Plating Procedure

1. Mix 10^6 phage with 20–100 µl of host cell culture.

2. Incubate for 15 minutes at 37°C or 25°C. This step can be omitted for P22.

3. Mix with 2.5 ml of soft LB agar (47°C) and spread onto a wet LB plate (freshly prepared).

4. Incubate right-side up at 37°C in a closed (wet) box.

III. Harvest

1. After confluent lysis (about 6 hr), cool plates by placing in cold room.

2. Overlay with 5 ml of cold λ dil (10 mM Tris [pH 7.5] and 10 mM $MgSO_4$) and keep in cold room overnight.

3. Remove overlay solution and add 2 drops of $CHCl_3$ (+EtOH). A crude phage stock can be stored at this stage at 5°C for several years or put into long-term storage (see Appendix 3). For λ DNA preparation, be careful to avoid contaminating the phage solution with bits of agar, and proceed to the next step.

4. To remove bacterial debris, centrifuge at low speed (about 10,000 rpm for 10 min) in a Sorvall SS-34 or a Beckman JA-20 rotor.

5. To sediment phage, centrifuge at 20,000 rpm for 3 hours.

6. Resuspend phage in 1 ml of λ dil (see Discussion). Transfer to a 1.5-ml Eppendorff microfuge tube and sediment suspension for 5 seconds to remove traces of debris. Stocks at this stage can be used to make DNA, or they can be stored.

7. To purify further (and make more stable stocks), follow Procedure 4-IA.

   One plate yields about 4 ml of 2×10^{10} particles/ml or about 10 μg of DNA after purification.

Discussion

I. See Procedure 1.

II. 1. The number of phage and volume of plating cells depends upon the plaque size. The highest-titer plate stocks are those containing plaques that just overlap to give confluent lysis. Too much overlap kills the cell lawn too early giving a very clear plate and low phage yield. If the plaques do not overlap, too few cells are infected and the uninfected cell density becomes too high, giving low phage yield. However, the exact ratio of phage to cells is not critical.

2. The incubation at high concentration of phage and cells assures good adsorption.

3. The agar should not be cooled below 47°C or it will start to solidify. If it is much hotter, it will kill many of the cells. If the agar is not melted completely, the plate will have a grainy appearance after incubation and plaques will be poorly defined. The bottom agar in the plates should be poured thick and should be wet so the plaques will run together, assuring confluent lysis.

4. Incubation of the plates right-side up will cause condensation to drip onto the lawn, aiding confluent lysis. Generally, we incubate in a large covered plastic box with wet paper towels in the bottom.

III. 1. (Steps 1 and 2) Harvesting the plates before or several hours after confluent lysis gives lower-titer stocks. The exact time for confluent lysis depends upon the plaque size, the number of phage and cells, and the growth rate of the cell. With a healthy phage and use of the conditions above, the time is about 6 hours.

With phage giving small plaques, the time may be extended to 8 hours. Cooling the plates increases the solidity of the top agar so that it will not be dislodged upon addition of the overlay liquid. Storing the plates overnight following overlaying allows the phage to diffuse out of the top agar into the overlay liquid.

2. (Steps 3, 4, and 5) The chloroform is added to kill all of the cells and prevent bacterial growth upon long storage. Ethanol is added (a few drops per pint of chloroform) to prevent oxidation products from accumulating in the chloroform. The plating stock can be stored at this stage. If these stocks are placed in airtight tubes, they can be stored for about 5 years. If DNA is to be prepared from purified phage, be very careful not to remove any agar. It is a potent inhibitor of many DNA enzymes. Also, it is not easily removed by CsCl gradient purification, since some agar has the same density as phage and has a high sedimentation coefficient. Some of the agar contamination, if present, may be removed during the centrifugation before CsCl purification. Do not sediment the phage longer than the clearing time. Overcompacting the phage can damage the particles, causing eventual loss of DNA.

3. (Step 6) The phage is very easily resuspended if not overcompacted. Nevertheless, it is good practice to allow the pellet to soak for a few hours in the cold before physical resuspension. A second, very short sedimentation will remove remaining cell debris and agar without loss of phage. For DNA preparation, step 6 must be followed if there is any agar contamination.

PROCEDURE 3

QUICK METHOD FOR THE PREPARATION OF P22 TRANSDUCING PHAGE

1. Inoculate 1.0 ml of LB broth with the desired donor strain and grow to full density. After overnight incubation at 37°C, there should be 2×10^9 cells/ml.

2. Infect with phage P22 (HT, int$^-$) at an moi of 0.01–0.1 phage/cell. A convenient method is to add 4 ml of P22 Broth to 1 ml of overnight culture. P22 Broth is LB broth containing full-strength (1×) E salts, 0.2% glucose, and 5×10^6 pfu/ml of phage P22 (HT, int$^-$). This phage-containing medium is fairly stable at room temperature. The P22 mutant used performs general transduction with increased frequency (HT; Schmieger 1972) and does not form stable lysogens (int$^-$).

3. Incubate 5 to 18 hours at 37°C with agitation. The culture may not clear.

4. Remove cells and debris by low-speed centrifugation.

5. Pour the supernatant into a tube containing 0.5 ml of chloroform and mix with a Vortex mixer.

6. Titer lysate (see Procedure 1). It should contain from 10^{10}–10^{11} phage/ml.

With phage giving small plaques, the time may be extended to 8 hours. Cooling the plates increases the solidity of the top agar so that it will not be dislodged upon addition of the overlay liquid. Storing the plates overnight following overlaying allows the phage to diffuse out of the top agar into the overlay liquid.

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6. Titer lysate (see Procedure 1). It should contain from 10^{10}–10^{11} phage/ml.

Discussion

It should be noted that a single plaque of phage P22 suspended in 4 ml of LB broth yields a suspension of about 10^6 phage/ml. Such a suspension can therefore provide the phage input for preparation of a stock of phage from a single plaque of any P22 phage by the method outlined above.

Reference

Schmieger, H. 1972. Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. 119:75.

PROCEDURE 4

PURIFICATION OF PHAGE

These procedures can be done serially, or just one can be done followed by an equilibrium gradient.

I. CsCl Block Density Gradients for Beckman SW 50.1 Rotor

A. The best procedure for removal of proteins is to sediment phage into CsCl from the top of the tube.

1. In the bottom of a 1/2 × 2-inch Beckman cellulose nitrate centrifuge tube, place 1 ml of 5.0 M CsCl, 10 mM $MgSO_4$, 10 mM Tris (pH 8), and 0.1 mM Na_2 EDTA ($\rho = 1.6$).

2. Overlay with 3 ml of 3.0 M CsCl, 10 mM $MgSO_4$, 10 mM Tris (pH 8), and 0.1 mM Na_2 EDTA ($\rho = 1.4$).

3. Overlay with 1 ml of phage in λ dil (10 mM $MgSO_4$ and 10 mM Tris [pH 7.5]). If phage is already in CsCl ($\rho = 1.5$), dilute 0.5 ml of phage with 0.5 ml of λ dil first.

4. Centrifuge in SW 50.1 rotor at 30,000 rpm for 1 hour at 20°C.

5. Remove phage from side of tube with a 1-ml syringe and a 5/8-inch, 25-gauge needle. Remove no more than 0.5 ml.

B. The best procedure for removal of DNA and RNA is to float the phage up into CsCl from the bottom of the tube.

1. Place 0.5 ml of phage, suspended in buoyant CsCl (i.e., $\rho \cong 1.5$), in the bottom of a cellulose nitrate centrifuge tube.

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5. Remove phage from side of tube with a 1-ml syringe and a 5/8-inch, 25-gauge needle. Remove no more than 0.5 ml.

B. The best procedure for removal of DNA and RNA is to float the phage up into CsCl from the bottom of the tube.

1. Place 0.5 ml of phage, suspended in buoyant CsCl (i.e., $\rho \cong 1.5$), in the bottom of a cellulose nitrate centrifuge tube.

2. Add an equal volume (0.5 ml) of saturated (25°C) CsCl solution (7.2 M) in 10 mM $MgSO_4$, 10 mM Tris (pH 8), and 0.1 mM Na_2 EDTA (ρ = 1.92), and mix well.

3. Overlay 3 ml of 5.0 M CsCl in 10 mM $MgSO_4$, 10 mM Tris (pH 8), and 0.1 mM Na_2 EDTA (ρ = 1.6).

4. Overlay 1 ml of 3.0 M CsCl in 10 mM $MgSO_4$, 10 mM Tris (pH 8), and 0.1 mM Na_2 EDTA (ρ = 1.4).

5. Centrifuge in SW 50.1 rotor at 30,000 rpm for 1 hour at 20°C.

6. Remove phage from side of tube with a 1-ml syringe and a 5/8-inch, 25-gauge needle. Remove no more than 0.5 ml.

II. Equilibrium Gradient for SW 50.1 Rotor

1. Transfer phage suspended in 4.0 M CsCl, 10 mM $MgSO_4$, 10 mM Tris (pH 8), and 0.1 mM Na_2 EDTA (buoyant CsCl) to a 1/2 × 2-inch Beckman cellulose nitrate centrifuge tube.

2. Centrifuge in SW 50.1 rotor at 30,000 rpm for at least 16 hours at 20°C.

3. Remove phage from side of tube with a 1-ml syringe and a 5/8-inch, 25-gauge needle.

4. Remove no more than 0.5 ml.

Discussion

I. A. Both λ and P22 phages have a density of about 1.5 g/ml. The exact density of λ depends on the DNA content; density increases with increasing size of DNA. The block gradient contains two layers of CsCl solution, with densities of 1.6 g/ml and 1.4 g/ml. Phage will sediment to the interface of these two solutions. Marking the interface with an ink pen during the layering of the solutions aids in locating the phage after centrifugation. The contaminating proteins have a density of about 1.3 g/ml and should float on the 1.4-g/ml CsCl solution. However, DNA and RNA have a greater density than the phage and may be found throughout the gradient.

B. This is a reverse block gradient, where the phage is floated again to a density interface. A density of about 1.7 g/ml is achieved by mixing equal volumes of phage in buoyant CsCl (ρ = 1.5) with saturated CsCl. The contaminating DNA (ρ = 1.7) and RNA (ρ = 1.9) should be removed by this flotation procedure.

II. This is a fast procedure to set up, but it requires longer centrifugation time. Contaminating RNA, DNA, and some proteins will not come to equilibrium in 16 hours and are distributed throughout the gradient. The advantage of this approach is that the phage comes to an equilibrium density in a gradient that is far less steep than that in the above procedure. As a result, if the phage preparation is heterogeneous with phage containing DNA of different size, more than one phage band will be observed.

The best method for observing a faint phage band is to illuminate the tube from above with a collimated light beam from a microscope light. A flat black background also aids visualization.

BURST SIZE

Oops! Many things including experiments could burst or explode in the lab, but the "burst size" is the number of bacteriophages produced in a single bacterial host cell.

PROCEDURE 5

Tn*10* TRANSPOSITION

I. Production of Defective Transducing Phage from Strain NK337

Defective transducing phage carrying Tn*10* are made by induction of strain NK337. This complicated lysogen (see Discussion) is extremely unstable and should be stored at -70°C or lyophilized.

1. Single-colony-isolate strain NK337 and check for temperature sensitivity. (This is evidence that the *c2*ts lysogen is present.) The test is done by checking single-colony-forming efficiency at 30°C and 42°C.

2. Grow a 30-ml overnight culture of NK337 at room temperature (less than 30°C).

3. Dilute culture by adding the entire 30 ml to 1500 ml of superbroth (see Appendix 1). Grow superbroth culture to ~3×10^8 cells/ml (OD_{650} = 0.5; remember to use a superbroth blank) at 30°C or less.

4. Shift culture to 39°C in an agitating waterbath.

5. Incubate with agitation for 3 hours or until lysis is observed.

6. Add chloroform (20 ml), agitate, wait, agitate. (It seems difficult to lyse cells in superbroth with chloroform. Repeated cycles of agitation and waiting for a few minutes over a period of 15 minutes seems to work well.)

7. Centrifuge at low speed to remove debris (sometimes several spins are required); save the supernatant. Most preparations lyse well and are clear after one spin.

8. Add tails (see below for procedure).

9. Concentrate phage particles by high-speed centrifugation (see Procedure 2).

10. Phage titer may be estimated by transducing TR4368 (his-644 [P22 sieA27]) to Tet^R. This transduction estimates the number of functional particles carrying the Tn10 element. The element can be transduced into the recipient's prophage since phage homology is present on both sides of Tn10. The particle titer is about 10^3 × the number of Tet^R transductants observed in this test.

11. A 1.5-liter preparation yields ~5×10^{12} particles after tail addition (see below).

II. Adding Tails to P22 Heads

A high proportion of phage particles released following induction of a P22 lysogen are deficient in tail parts. These may be added to the particles by the procedure outlined below (based on methods of Israel et al. 1967).

The procedure involves preparing a lysate of a head-defective mutant. The phage also carries a 13^- mutation, which prevents lysis and promotes high production of tail parts. The strain for making P22 tails is P22-503 (c1-7 12amN114 13amH101), and the Salmonella hosts are TR248 (cysA1349am hisC527am) and TR251 (cysA1349am hisC527am supD [$su1^+$]).

A. Preparation of Tails

1. Grow a stock of phage P22-503 on host strain TR251 (Procedure 2 or 3).

2. To check revertant level, plate on TR251 (permissive for

the amber mutations) and TR248 (restrictive for the amber mutations).

3. Grow a 2-liter culture of TR248 in LB broth to ~2×10^8 cells/ml at 37°C.

4. Infect with phage P22-503 at an moi of 5 phage/cell.

5. Following infection, incubate 90 minutes at 37°C with agitation.

6. Collect infected cells by centrifugation (10 min at 6000 rpm) and resuspend in saline (1/100 of original volume).

7. Lyse cells by addition of chloroform and vigorous agitation.

8. Treat with DNase (10 μg/ml) for 10 minutes at room temperature.

9. Centrifuge preparation 15 minutes at 5000 rpm to remove debris.

10. Centrifuge 2 hours at 17,000 rpm to remove any complete phage particles. Repeat this step. In each case save the supernatant and discard the pellet.

11. Assay the supernatant by plating on TR251 for intact phage. There should be less than 10^4 pfu/ml.

B. Attaching Tails to Heads

Mix head and tail suspensions and incubate 2 hours at 30°C. Follow the titer increase by assaying the phage. Use the yield of tail parts from 2 liters of culture to saturate the ~5×10^{12} heads obtained by induction of NK337 (1.5 liters).

III. Transposition Transduction

1. Grow recipient cells to 5×10^8 cells/ml in LB broth.

2. Concentrate cells tenfold by centrifugation (10 min at 5000 rpm; resuspend in 1/10 volume).

3. Infect with phage at an moi of 0.8 particle/cell. Adsorb for 30 minutes at 37°C in LB broth.

4. Plate infected cells onto Green + Tet (25 μg/ml) + EGTA (10 mM) plates. Incubate plates at 41°C. Pick colonies and screen for mutants.

5. Tet^R clones should be found at a frequency of $1/10^5$–$1/10^6$ infected cells. Following the above procedure, and assuming phage is added in a very small volume, 0.1 ml of infected cells gives 200 Tet^R colonies. Approximately 1% of the Tet^R clones are auxotrophic.

Discussion

I. The procedure for producing defective transducing phage is as described by Kleckner et al. (1975). The lysogenic strain NK337 (described below) is induced to produce phage by exposure to high temperature.

NK337: hisC527 leu-414 supE (P22 c2ts29 12amN11 13amH101 int-3 Tn10)

The amber suppressor suppresses the phage's 12^- and 13^- mutations. Induction occurs at 40°C due to the c2ts29 mutation. The phage's int^- mutation causes a high frequency of aberrant prophage excisions; therefore, many of the genomes are defective, but they do include the Tn10 element.

II. The basic procedure for the transposition transduction is to infect cells with DNA carrying Tn10 so that the transposable element cannot be integrated either by lysogeny or by standard recombination. The phage carrying Tn10 is c2ts, so no repression is possible at high temperature. The phage is 12^-13^-, so very little replication of complete phage occurs in su^- recipients. The int^- mutation causes much of the lysate to be defective due to aberrant excision in phage preparation. (See sections above on the growth and tailing of the defective P22 phage.)

In the recipient, the int^- mutation also prevents integration of any genomes that escape the other restrictions. Homologous recombination is prevented, since Tn10 is integrated into the phage genome and the recipient possesses no phage material.

EGTA is added to transduction plates because it chelates Ca^{++} and prevents phage adsorption. This prevents multiplication of viable phage on the transduction plate. Green plates are not essential but they are inexpensive and permit observation of colonies in which phage is multiplying (colonies are dark green). Uninfected colonies and stable lysogens form light-colored colonies.

References

Israel, J.V., T.F. Anderson, and M. Levine. 1967. In vitro morphogenesis of phage P22 from heads and base-parts. Proc. Natl. Acad. Sci. 57:284.

Kleckner, N., R. Chen, B.-K. Tye, and D. Botstein. 1975. Mutagenesis by insertion of a drug-resistance element carrying an inverted repetition. J. Mol. Biol. 97:561.

PROCEDURE 6

SELECTION OF Tet^S MUTANTS FROM STRAINS CARRYING Tn10

1. Grow up an overnight culture of a strain carrying Tn10 in LB broth.
2. Dilute the culture and plate ~10^6 cells on Bochner selection medium.
3. Incubate plates at 37°C.
4. Pick colonies and single-colony-isolate.
5. Test for tetracycline resistance.

Discussion

In manipulating strains carrying Tn10, frequently it is useful to be able to select mutants that have lost tetracycline resistance. Such removal allows repeated use of Tn10 in strain construction and should also permit selection of Tn10-generated deletions and inversions. The selection system described was devised by Bochner et al. (1980). Although the detailed basis of the selection is not fully understood, some points of information follow:

1. Autoclaving chlortetracycline converts it to a compound that induces the Tn10 resistance function. This inducing substance is not toxic to cells.

2. Cells whose tetracycline resistance is induced are sensitive to growth inhibition by fusaric or quinaldic acid. Loss of the tetracycline-resistance determinant by mutation leaves cells resistant to fusaric or quinaldic acid.

3. Fusaric and quinaldic acid are lipid soluble chelators of divalent cations.

4. If iron or manganese is added to the medium, quinaldic and fusaric acid are no longer inhibitory.

5. It is critical to plate a low cell density ($<10^6$/plate).

Reference

Bochner, B., H.-C. Huang, G. Schieven, and B.N. Ames. 1980. Positive selections for loss of tetracycline resistance. J. Bacteriol. 143:926.

PROCEDURE 7

RED PLATE TEST FOR λ PHAGE WITH A FUNCTIONAL β-LACTAMASE GENE

The purpose of this assay is to allow one to distinguish visually between phages with and without a functional β-lactamase gene. The assay can be used to identify derivatives of a λ*amp* phage that have acquired a mutation affecting β-lactamase. The rationale for this procedure is discussed below.

1. Pour Red plates (Appendix 1) 1 day in advance.

2. Grow DB6430 and DB4383 to 3×10^8 cells/ml at 37°C in TYM broth. Do not store (even on ice) for very long before using.

3. Dilute phage until 0.1 ml of the appropriate dilution gives 40–100 plaques per plate. Add 0.1 ml of this dilution to 0.1 ml of DB4383 and let the mixture incubate at room temperature for 20 minutes.

4. Add 0.1 ml of DB6430 to this mixture and plate with 2.5 ml of λ top agar on Red plates. Incubate at 34°C.

5. Look at plates every few hours beginning 24 hours after plating.

6. It is important to check the method by plating control phages (λ*amp* alone, λ without *amp*, and a mixture of the two phages).

Discussion

The assay takes advantage of the fact that when β-lactamase is released into the medium, it destroys ampicillin and saves sensitive gal$^+$ cells in the vicinity. These rescued gal$^+$ cells ferment galactose, reduce tetrazolium, and form a red halo around the plaque. To assure growth of phage, gal$^-$ cells are added to the plating bacteria. These gal$^-$ cells are resistant to ampicillin (but do not release β-lactamase). The gal$^-$ bacteria can act as hosts for phage but reduce no tetrazolium (form no red color). Thus, the gal$^-$ Amp^R cells (DB4383) can grow everywhere on the plate but form no color; the gal$^+$ Amp^S cells (DB6430) grow (and form red color) only if saved by β-lactamase in the vicinity of a β-lactamase-producing (bla$^+$) λ plaque.

It is important to adjust the concentration of ampicillin in the plates for each lot of drug. The concentration should be the minimum that suppresses the growth of the DB6430 in the lawn.

PROCEDURE 8

HYDROXYLAMINE MUTAGENESIS

I. Isolating Mutants in Phage or Cloned Gene (e.g., β-lactamase gene)

1. Mix 0.4 ml of phosphate EDTA buffer (0.5 M KPO_4 at pH 6.0, 5 mM EDTA), 0.5 ml of sterile water, 0.8 ml of hydroxylamine solution (1 M NH_2OH at pH 6, made fresh by adding 0.56 ml of 4 M NaOH to 0.35 g of NH_2OH and then adding sterile water to 5 ml), 0.1 ml of sterile 0.2 M $MgSO_4$ (for λ), and 0.2 ml of phage stock (10^9–10^{11} pfu/ml).

2. Do a control (sterile water instead of hydroxylamine).

3. Incubate these mixtures at 37°C for 12–48 hours.

4. Take samples periodically (about every 6–8 hr) by diluting 1/100 into cold LBSE (LB made 1 M in NaCl and 1 mM EDTA). After 1 hour, dilute 2× into λ dil and store in the cold.

5. Titer each sample for plaques on a permissive host. Expect a few percent survival per 24 hours of incubation in hydroxylamine and 50–100% survival in the control. Plate some dilutions that will give 1000–3000 plaques; these can be scored for frequency of clear plaques. Save dilutions in the cold for large-scale plating later, since they are more stable than the LBSE solutions.

6. Isolate phage mutants by plating appropriate dilutions (~100/plate) on a permissive host and picking fresh (<18 hr) plaques into nonpermissive and permissive hosts (i.e., freshly prepared plates with appropriate plating bacteria in the top agar) with sterile toothpicks. Often it is desirable to sterilize the lawns before picking plaques. This is done by inverting the plate over a planchet containing chloroform for about 10 minutes.

II. Localized Mutagenesis by Cotransduction

1. Mix 1 ml of phosphate-EDTA buffer, 1.5 ml of sterile water, 2 ml of hydroxylamine, and 0.5 ml of P22 phage (2×10^{11}–2×10^{12} pfu/ml) that has been grown on a donor strain with a selectable marker linked to the region intended to be mutagenized specifically (Hong and Ames 1971).

2. Incubate 20–50 hours at 37°C.

3. Take samples periodically (every 6–8 hr) by diluting into cold LBSE.

4. Titer samples immediately for killing and for proportion of clear-plaque mutations (see Discussion).

5. After about 30–50 hours (or about 0.1–1% survival), centrifuge the mixture in a Sorvall rotor at 17,000 rpm for 2 hours to pellet the phage. Resuspend in 1 ml of LBSE by simply letting the LBSE wet the pellet (with occasional agitation) overnight. (Do not attack the pellet with a pipette.)

6. For transduction, use about 0.1 ml of mutagenized phage and 10^8 bacteria per plate. Plate should be selective for the selectable linked marker (such as Tet^R). When colonies appear, they should be replica-plated to find mutants carrying mutations linked to the marker selected (e.g., Tn10).

Reference

Hong, J.-S. and B.N. Ames. 1971. Localized mutagenesis of any specific small region of the bacterial chromosome. Proc. Natl. Acad. Sci. 68:3158.

Discussion

Hydroxylamine is a potent mutagen that can be used in vitro on DNA or DNA viruses. Its advantages include known specificity and mechanism of action. It produces G-to-A transitions exclusively. When used properly, it gives a very high ratio of mutagenic events to lethal events. It is thus a useful mutagen for circumstances (such as localized mutagenesis) where heavy mutagenesis is desired. By the same token, it should be kept in mind that heavy chemical mutagenesis frequently causes multiple mutations. With temperate phages, a convenient measure of the degree of mutagenesis is the number of clear-plaque mutants per survivor. It is a good practice to monitor the number of clear-plaque mutants even in localized mutagenesis experiments as lethality is not an infallible guide to the degree of mutagenesis.

I. A. For P22, the $MgCl_2$ should be replaced with sterile water. λ phages (especially those with oversize genomes) are sensitive to EDTA, and therefore $MgCl_2$ should be added. The small amount of EDTA in LBSE is not very lethal in the cold; the twofold dilution into λ dil restores Mg^{++} ions.

B. It is also useful to score the frequency of clear-plaque mutations when mutagenizing $\underline{c}^+$ phages. Clear plaques can be scored even on fairly crowded plates. Maximum achievable mutagenesis appears to be about 5% clear plaques.

C. A typical scheme for isolating amber mutations of phage genes might involve plating mutagenized phage on an $\underline{su}^+$ lawn at 30°C and picking the plaques onto three plates: one $\underline{su}^-$ (to be incubated at 40°C), one $\underline{su}^+$ (to be incubated at 40°C), and one $\underline{su}^+$ (to be incubated at 30°C). From such a scheme, one might get amber and temperature-sensitive mutants.

II. A. It is important to purify the new mutants as early as possible to free them of phage particles. In the case of P22, inclusion of

EGTA in media used to identify mutants will improve the chances of recovering phage-sensitive, nonlysogenic mutants.

B. See I-B above.

References

Hall, D.H. and I. Tessman. 1966. T4 mutants unable to induce deoxycytidylate deaminase activity. Virology 29:339.

Parkinson, J.S. 1968. Genetics of the left arm of the chromosome of bacteriophage lambda. Genetics 59:311.

PROCEDURE 9

SELECTION OF DELETION MUTANTS OF λ

1. Make a plate stock of EDTA-sensitive phage (see Discussion). Use λ dil without magnesium to elute the phage from the agar. Alternatively, dilute a normal stock 1/5 into λ dil without magnesium.

2. Dilute low-magnesium phage stocks into 10 mM EDTA (pH 7). If the stock has an inadequate titer (less than about 10^{10} phage/ml), one can add EDTA to the stock to a final concentration of 10 mM, being sure to account for any Mg^{++} in the initial solution.

3. Incubate at 48°C for 30 minutes. The titer should fall 10^4- to 10^5-fold. Incubation at higher temperature can be used to select larger deletions or to select deletions from marginally sensitive phage stocks.

4. Take 0.1 ml of the treated stock (at least 10^5 phage/ml) and use it to make a new plate stock. The method is given in Procedure 2. Use λ plates.

5. This new stock is treated to a second cycle of EDTA-heat exactly as in steps 1–3. The titer should fall about 10^3 or less.

6. Plate out second-cycle phage suspension for single plaques. These can be picked and tested individually for possession of mutations (as described in Procedure 8), or the suspension can be plated on indicator plates that permit direct identification of mutants (such as the Red plate test described in Procedure 7).

Discussion

Magnesium ions are required to maintain the structural integrity of the λ phage head. Removal of magnesium ions by chelating agents such as EDTA or pyrophosphate results in the bursting of any λ head with a DNA content greater than about 96% of wild type. As a result, naturally occurring deletions of λ can be selected from stocks by exposure to chelating agents. Several cycles of selection are usually required because natural variants of λ (λ*) exist in any population; these variants are resistant to chelating agents for reasons other than lower DNA content (probably increased protein content). These further cycles usually can be carried out by plating on plates containing chelating agents. For further information on this procedure, see Parkinson and Huskey (1971).

It is critical to start with phage having a genome size large enough to be sensitive to a chelating agent such as EDTA. This is most easily determined empirically on plates containing EDTA. Screen phage by overstreaking on lawns of E. coli on trypticase EDTA (1 mM). (The concentration of EDTA may need to be varied depending upon the lot of trypticase or agar.) Use top agar, which also contains trypticase and EDTA. In parallel with the tests on EDTA-containing medium, streak the phage on standard λ plates with λ top agar. Do control streaks of λ^+ (wild type) and λcI857 b515 b519 nin5 intam29 on each plate. Compare the sizes of plaques on each plate after incubation overnight at 42°C. EDTA-sensitive phage will give pinpoint plaques (or no plaques at all). It is important to score isolated plaques, since spot tests are not reliable guides.

Reference

Parkinson, J.S. and R.J. Huskey. 1971. Deletion mutants of bacteriophage lambda. Isolation and initial characterization. J. Mol. Biol. 56:369.

PROCEDURE 10

PHAGE RECOMBINATION AND COMPLEMENTATION TESTS IN VIVO

I. Standard Cross (to determine recombination frequency or to construct a recombinant)

1. Grow an overnight culture of a bacterial host permissive for both phages you want to cross. Titer each phage accurately (Procedure 1).

2. Dilute culture 1/1000 in TYM (λ crosses) or LB (P22 crosses) broth and grow to a density of no more than 2×10^8 cells/ml (Klett = 40, or use a Petroff-Hausser chamber).

3. Sediment and resuspend in cold λ dilution media (for λ crosses) or buffered saline (for P22 crosses) to obtain a density of 4×10^8 cells/ml.

4. Dilute each parental phage to a density of 4×10^9 phage/ml. Mix together 1:1.

5. Add 0.2 ml of phage mixture to 0.2 ml of cells and allow to adsorb at 37°C for 15 minutes.

6. Dilute 10^4-fold into LB (not TYM) broth prewarmed to 37°C and incubate for 90 minutes, and then add 2 drops of chloroform.

7. Titer under conditions permissive for both parental phages (to obtain total progeny titer) and nonpermissive for either parent (to obtain recombinant titer). Recombination frequency = (2 × nonpermissive titer) ÷ total progeny titer.

II. Complementation Test for Phage Growth

1. Grow an overnight culture of a bacterial host nonpermissive for the mutant phage. Then continue with steps 1–6 of the standard cross, taking care to maintain nonpermissive conditions during steps 5 and 6.

2. Titer total progeny on permissive host under permissive conditions.

III. Spot Test for Complementation of Phage (λ or P22) Mutants

Phage mutants can be screened rapidly pairwise for complementation using a spot test on plates seeded with a nonpermissive host. A convenient method involves adsorption of one of the pair to be tested to the nonpermissive cells in the top agar, allowing the agar to harden, and then spotting the other phage(s) on top. The spot test is much more reliable if each of the mutants is adsorbed in turn to the cells so that reciprocal pairs of tests of each pair of mutants are done. Often it may be useful to test several dilutions of each phage.

1. In LB broth grow an overnight culture of a nonpermissive bacterial host.

2. Dilute the cells 1/50 into TYM (for λ) or LB (for P22) broth and grow them to a density of less than 2×10^8 cells/ml.

3. Add between 10^7 and 10^8 of the phage to be tested to 0.2 ml of the bacterial cells. Add 2.5 ml of molten λ top agar and plate on a λ plate. Preadsorption is not necessary. Prepare a plate with no phage as a control.

4. After the agar has hardened (about 10 min), spot, from a sterile capillary, about 20–50 μl of the various phage stocks to be tested.

For best results use a dilution series for each stock: 10^{-2}, 10^{-3}, and 10^{-4} dilutions of stocks at about 10^{10} phage/ml will cover the useful range. Include wild type and the phage adsorbed to the cells as positive and negative controls on each plate.

5. Incubate overnight at 37°C (or higher if a temperature-sensitive mutant is involved).

Complementation has occurred if, even at high dilutions, lysis is observed beyond that observed in the relevant controls. Sometimes phage at high concentration (low dilution) can kill cells on which they are unable to grow. Only the controls stand between you and confusion. Several dilutions are always helpful.

The spot test is only a guide: Real confirmation of complementation requires in most cases direct test of growth after coinfection in liquid culture.

Discussion

I. 1. Phage crosses are carried out by adsorbing two phages of different genotypes to the same permissive host. Any cell with good recombination functions can be used. C600 contains supE, and BNN45 contains both supE and supF, and is hsdR$^-$ hsdM$^+$, and therefore unmodified amber mutant phage can be crossed in the latter host.

2. Recombination seems to be higher in exponentially growing cells. Low-level UV irradiation before the phage cross will also increase recombination.

3. Concentrating the cells assures good phage adsorption.

4. (Steps 4 and 5) The intention is either to measure the frequency of recombinants or to construct a strain recombinant for some of the markers carried by the two parents. In either case, the techniques are the same. The importance in using as permissive conditions as possible for either purpose cannot be stressed too strongly. Bias in the crosses caused by selection can easily make interpretation of the result impossible or make the frequency of a desired recombinant inconveniently low. The moi is a very important factor. It should be high enough to insure relatively equal input to every cell, but not so high as to interfere with growth. Usually an moi of five to seven phage of each parental type per cell is used. Titering just a day or two in advance of the cross is recommended.

5. (Step 6) The cells are diluted to give good aeration for continued growth and to prevent readsorption following cell lysis. Use of LB broth (which contains no maltose) for this step further inhibits readsorption of progeny λ phage after lysis.

6. (Step 7) The criteria for a good cross are burst size (should be more than 20), equal transmission of parental markers, and good

adsorption of the input phage. It is usual to measure all of these parameters in crosses intended to produce recombination frequencies. When recombination frequencies are low, it is essential to know the frequency of revertants expected. The usual control for this are mock infections using only one parent at a time.

II. 1. Liquid complementation tests are run almost exactly like crosses, except that nonpermissive bacteria are used as hosts. Here again it is important to use an equal input (moi = 5–10) and to measure unadsorbed phage, cell numbers at time of infection, as well as the burst size, which, in this case, is the aim of the exercise. Often antiphage serum is used to reduce the background of unadsorbed phage. The mock infections of each parent alone are very important in liquid complementation, as are measurements of recombination frequency under the nonpermissive condition, since either reversion or recombination can result in surprisingly large bursts.

The important idea in complementation is to carry out adsorption and infection under strictly nonpermissive conditions. For example, to test a temperature-sensitive mutant against an amber mutant, one uses an su^- host at high temperature. The temperature of adsorption and growth must therefore be controlled carefully. A wild-type infection and separate mutant infections under the same conditions are essential controls.

2. Titering must be on a host permissive for both parents (e.g., su^+ at 25°C in the example of complementation between a temperature-sensitive and an amber phage). Results are derived from comparison of growth yield of the coinfection with the separate mutant and wild-type control infections.

GEDANKEN EXPERIMENT

For those thinkers who do not work in the laboratory, the Gedanken experiments are a way out. Some Gedanken experiments are real contributions and some we should better forget.

PROCEDURE 11

EXTRACTION OF DNA FROM PHAGE λ

I. Formamide Method

A. 1. Mix 1 volume unit of phage in buoyant CsCl, 10 mM $MgSO_4$, 10 mM Tris (pH 8), and 0.1 mM Na_2 EDTA with 1/10 volume unit 2 M Tris and 0.2 M Na_2 EDTA (pH 8.5) in a polypropylene microfuge tube.

2. Add 1 volume unit of formamide and let stand 30 minutes to several hours at room temperature.

3. Add 1 volume unit of H_2O.

4. Add 6 volume units of EtOH at room temperature.

5. Sediment DNA precipitate for 2 seconds in microfuge.

6. Discard supernatant and rinse precipitate with 70% EtOH.

7. Discard rinse solution and dissolve dry precipitate in 10 mM Tris (pH 7.5) and 1 mM Na_2 EDTA.

8. Increase salt concentration for long-term storage.

B. 1. Mix phage in buoyant CsCl, 10 mM $MgSO_4$, 10 mM Tris (pH 8), and 0.1 mM Na_2 EDTA with 1/10 volume unit 0.2 M Na_3 EDTA.

2. Dialyze against 50% formamide, 0.2 M Tris (pH 8.5), and 0.02 M Na_2 EDTA for 12–24 hours at room temperature.

3. Dialyze against DNA storage buffer, 0.1 M NaCl, 0.05 M Tris (pH 8.5), and 0.01 M Na_2 EDTA; or, for endonuclease EcoRI cleavage, against 0.1 M NaCl, 0.05 M Tris (pH 7.4), and 0.1 mM Na_2 EDTA.

4. Requires four buffer changes at 200-fold dilution.

References

Thomas, M. and R.W. Davis. 1974. Studies on the cleavage of bacteriophage lambda DNA with EcoRI restriction endonuclease. J. Mol. Biol. 91:315.

St. John, T. (pers. comm.)

Discussion

Generally, λ DNA is obtained from phage by phenol extraction followed by removal of dissolved phenol by ether extraction. We have found that DNA with fewer single-strand nicks and higher infectivity can be obtained by formamide extraction. The phage proteins, as phage ghosts, are probably not removed. However, we have not observed any interference using most enzymological techniques. Formamide treatment of phage appears to cause the DNA in the phage to be ejected from the tail.

A. 1. The Na_2 EDTA is added to chelate the Mg^{++}

2. The ejection of DNA seems very fast. However, long-term storage in formamide does not appear to be deleterious to the DNA.

3. The water is added so that the CsCl will not precipitate upon addition of ethanol.

4. (Steps 4 and 5) The DNA is precipitated rapidly, and generally it is not necessary to cool the solution. If sedimentation is not very short, the DNA cannot be resuspended readily.

5. (Step 6) The precipitate is rinsed to remove residual formamide and CsCl.

6. (Steps 7 and 8) DNA dissolves fastest in very low salt. However, for long-term storage (months), DNA shows less degradation in higher salt and Na_2 EDTA concentrations.

B. This procedure is similar to that above except it uses dialysis to add and remove the formamide. It does not require an ethanol precipitation. There may be impurities in the formamide that are not removed by ethanol precipitation. Presumably, anything that will dialyze in will also dialyze out. This procedure is probably best suited for the preparation of large amounts of λ DNA, since a large ethanol precipitate of DNA may be difficult to redissolve.

II. Rapid λ DNA Isolation

1. Make a normal λ plate stock using media prepared with agarose.
2. Cool the plates and then overlay them with 5 ml of cold λ dil. Let plates sit overnight at 4°C.
3. Pipette 0.4 ml of the clear lysate into a 1.5-ml microfuge tube. The remainder of the lysate can be saved for culture purposes.
4. Add 1 µl of diethyloxydiformate at room temperature.
5. Add 10 µl of 10% SDS. Invert tube to mix.
6. Add 50 µl of 2 M Tris and 0.2 M Na_2 EDTA (pH 8.5) and mix.
7. Incubate at 70°C for 5 minutes in the hood.
8. Add 50 µl of 5 M KAc (potassium acetate).
9. Cool tubes and wait at least 30 minutes with tubes in ice.
10. Sediment precipitate in microfuge for 15 minutes.
11. Decant supernatant into new tube.
12. Fill tube with EtOH at room temperature.
13. Sediment precipitate in a microfuge for 5 minutes.
14. Discard supernatant and invert tube on paper towel to drain. All liquid should either drain off or evaporate, but do not overdry.
15. Dissolve precipitated DNA in 50 µl of:

    10 mM Tris (pH 7.5)
    1 mM Na_3 EDTA
    10 µg/ml of RNase A

16. 5 µl is usually sufficient for one restriction digest and gel track.

Discussion

1. Unpurified agar is a potent inhibitor of most restriction endonucleases and other DNA enzymes. The inhibitor may be sulfonated carbohydrates. The λ DNA recovered with this procedure is from phage and from free DNA released into the media. Upon endonuclease cleavage and electrophoresis, the E. coli DNA is spread over the entire gel track, which allows visualization of unique λ DNA bands that are in higher molar amounts.

2. Cooling the plates increases the solidity of the agarose, which prevents its becoming suspended during the overlaying and removal in the next step. The phage and phage DNA diffuse out of the top agarose into the overlay liquid. The cold plates and cold λ dil also appear to decrease the DNA degradation once it diffuses into the overlay liquid.

3. The agarose plate stock should give a normal λ plating stock of 4 ml of 10^{10} phage/ml. The 0.4 ml contains enough phage and phage DNA for about ten gel tracks.

4. The diethyloxydiformate is used to inactivate nucleases. Presumably, the nucleases are inhibited initially by the agarose, since the DNA is not degraded at 37°C until after it is removed from the plate in the overlay liquid.

5. The SDS (sodium dodecyl sulfate) denatures the proteins and releases DNA from the phage.

6. The Tris is added to buffer the solution, since the diethyloxydiformate generates about 30 mM CO_2. The Na_2 EDTA is added to chelate the Mg^{++} from the λ dil.

7. The heating is used to complete the protein denaturation.

8. The addition of KAc causes a potassium-dodecyl sulfate precipitation

(K-DS ppt) of the denatured proteins. The KAc is added to the tubes before cooling so that the precipitate will form more slowly and be less flocculent. Such precipitates are more readily removed by centrifugation.

9. The cooling assures complete precipitation.

10. If some of the precipitate does not sediment, flick the tube to remove air trapped in the precipitate and resediment.

11. Try not to remove any precipitate. If the supernatant appears cloudy, resediment.

12. The sample contains high-molecular-weight DNA and is easily precipitated with ethanol. The sample should not be cooled, as undesirable contaminants also precipitate at lower temperatures.

13. (Steps 13 and 14) The KAc is very soluble in ethanol, so no salt precipitation will occur. It is important that all the liquid drain off in order to eliminate the soluble contaminants and the KAc. If the tubes fail to drain, they can be swabbed with a tissue or cotton-tipped applicator.

14. (Step 15) RNase is added to remove contaminating RNA that can inhibit DNA enzymes. The digested RNA need not be removed.

Reference

Cameron, J.R., P. Philippsen, and R.W. Davis. 1977. Analysis of chromosomal integration and deletions of yeast plasmids. Nucleic Acids Res. 4:1429.

III. Preparation of DNA from Temperature-inducible Sam7 Lysogens

1. Streak lysogen for single colonies and incubate at 30–32°C.

2. Check growth of several colonies at 32°C and 42°C.

3. Pick one colony from the 32°C plate that does not grow at 42°C, and grow the overnight culture in LB broth at 32°C.

4. Inoculate 1000 ml of LB broth (pH 7.5–8) with 10 ml of the overnight culture.

5. Grow at 32°C until A_{600} = 0.6 (about 3–4 hr).

6. Heat at 42–43°C and continue growth at this temperature for at least 15 minutes.

7. Grow at 37°C for an additional 3 hours (maintain pH 7.5–8; if acidic, add NaOH).

8. Add 5 ml of $CHCl_3$ and agitate at 37°C for 10 minutes (lysis occurs after a few minutes).

9. Spin down cell debris (7000 rpm, 5 min) in a JA-7.5 rotor.

10. Spin down phage (7000 rpm, 15 hr) in a JA-7.5 rotor.

11. Resuspend phage pellet in 9 ml of 4 M CsCl (ρ = 1.5), 10 mM $MgSO_4$, and 0.1 mM Na_2 EDTA.

12. Fill two Beckman SW 50.1 tubes and band phage at 30,000 rpm (20°C) for at least 16 hours.

13. Extract the band by side puncture with a 25-gauge syringe needle; remove 0.5 ml from each tube.

14. Store phage in CsCl at 4°C. (For long-term storage, the DNA is preserved best in the phage.)

Storage of cells: Grow overnight culture at 32°C, make the culture 7% v/v in dimethylsulfoxide, and store frozen at -70°C.

IV. λ DNA Strand Separation

A. Denaturing Solution

1. At 0°C, in a 1/2 × 2-inch Beckman polyallomer centrifuge tube, add 1.0 A_{260} unit (50 μg of DNA) of λ phage (maximum volume = 200 μl; minimum A_{260} of stock = 5.0).

2. Add 200 μl (minus phage volume) of H_2O.

3. Add 5 μl of 1 M Na_4 EDTA.

4. Add 5 μl of 4% sodium N-lauroyl sarcosinate in 10^{-2} M Tris (pH 7.5).

5. Add 35 μl of 1 M NaOH.

6. Let stand 10–30 minutes at room temperature.

B. Quenching Solution

1. Add 40 μl of 2 M Tris-HCl to a 1.5-ml microfuge tube.

2. Add 1.0 A_{260} unit poly(rUG) (e.g., 7 μl at A_{260} = 175) in H_2O.

3. Add 245 μl (minus poly[rUG] volume) of H_2O. Total volume of solutions A and B = 0.530 ml.

4. Place both solutions (A and B) in ice and cool to 0°C. Inject quenching solution (B) from pipetter into DNA solution (A) in polyallomer tube on Vortex mixer at position 3. Place back in ice.

C. Add 2.13 ml of 7.29 M CsCl (ρ^{25} = 1.91) (η^{25} = 1.4188).

Centrifuge: 30,000 rpm at 10°C for 48 hours in a Beckman SW 50.1 rotor.

Fractionation: 8-drop fractions, total of 40 fractions.

References

Davis, R.W. and R.W. Hyman. 1971. A study in evolution: The DNA base sequence homology between coliphages T1 and T3. J. Mol. Biol. 62:287.

Summers, W.C. and W. Szybalski. 1968. Totally asymmetric transcription of coliphage T7 in vivo: Correlation with poly G binding sites. Virology 34:9.

PROCEDURE 12

ISOLATION OF PLASMID AND BACTERIAL DNA

I. Large-scale Isolation of E. coli Plasmid DNA

1. Start from a single colony of E. coli tested for the presence of the plasmid (Tet^R, Amp^R, Cam^R, etc.). Grow 100 ml of LB broth culture to saturation in a 1-liter flask with vigorous agitation. Yield: ~50 μg. (Preparation below is for one tube of an SW 50.1 rotor. See page 117 for volumes for other rotor tubes.)

2. Sediment cells at 8000 rpm for 5 minutes at 5°C.

3. Resuspend cells in 1/4 volume 10 mM Tris (pH 8.5) and 1 mM Na_3 EDTA and resediment.

4. Resuspend cells in 2 ml of 15% sucrose, 0.05 M Tris (pH 8.5), 0.05 M Na_2 EDTA, and 1 mg/ml of freshly prepared lysozyme and transfer to a 10-ml centrifuge tube that can be centrifuged to 20,000 rpm. Incubate at room temperature for 10–60 minutes.

5. Add 2 ml of Triton solution (0.1% Triton X-100 [Sigma], 0.05 M Tris [pH 8.5], and 0.05 M Na_2 EDTA). Incubate at room temperature for 10–20 minutes. If lysis does not occur and the suspension does not become very viscous, incubate at 37°C for 30 minutes.

6. Spin at 20,000 rpm for 1 hour in a JA-21 rotor at 5°C.

7. Decant supernatant by pouring into a graduated test tube until the very viscous material above the pellet is reached. Usually this is less than the last half inch of clarified supernatant. Sometimes the supernatant is relatively nonviscous down to the pellet; in this case, take it all.

8. Adjust volume to 4.0 ml. Add 3.7 g of solid CsCl and 0.4 ml of 10 mg/ml of ethidium bromide (Sigma). Refractive index should be between 1.390 and 1.396 or a density of 1.59 g/ml. The density is the most reliable and can be determined easily by weighing a known volume. This mixture will just fill one SW 50.1 rotor tube.

9. Centrifuge at 35,000 rpm for 48 hours at 20°C.

10. View bands by illuminating the tube with long-wavelength UV. The lower band contains the covalently closed circular DNA. Collect the DNA by puncturing the side of the tube with a 20- to 21-gauge syringe needle.

    Relaxed DNA: ρ = 1.55 g/ml
    Native superhelical DNA: ρ = 1.59 g/ml

For larger culture volumes, use a fixed-angle or vertical rotor.

Rotor	50 Ti	60 Ti	60 Ti	60 Ti	60 Ti
Volume of culture (liters)	0.1	0.8	1.5	3	6
Number of rotor tubes	1	1	2	4	8
Lysozyme solution (ml)	4.5	13.5	27	54	108
Triton solution (ml)	4.8	14.5	29	58	116
Adjusted supernatant (ml)	8.7	27.5	55	110	220
CsCl (g)	8.3	26.13	52.26	104.5	209
Ethidium bromide solution (ml)	0.9	2.75	5.5	11	22

Discussion

1. E. coli can become cured of many plasmids, even multicopy plasmids, on long-term storage on plates, in stabs, or in broth. Therefore, verification of the presence of the plasmid is advisable just before large-scale plasmid isolation. However, the large-scale culture need not always contain a selective nutrient or drug to assure maintenance of the plasmid.

2. (Steps 2 and 3) This is to wash the cells free of culture media and other soluble components (Mg^{++}, salts, buffers, etc.) that might inhibit the following steps.

3. (Step 4) The Na_2 EDTA is used to remove Ca^{++} from the cell wall, which allows more accessibility by the lysozyme. The lysozyme is somewhat unstable at alkaline pH, which is the reason for using a freshly prepared solution. At pH 4–5, it is stable for several weeks at 5°C and for a few days at room temperature. The enzyme may be dissolved in cold distilled water.

4. (Step 5) Triton X-100 is a nonionic detergent and should cause the lysozyme-treated cells to lyse. Most of the large cellular DNA is not released from the cell debris and will sediment to form part of the pellet. Care should be taken not to mix too vigorously so that the cellular DNA is not released.

5. (Step 6) The pellet should appear somewhat gelatinous and the supernatant somewhat yellow. A clear, colorless supernatant and a compact, opaque pellet indicate that the cells have not lysed. If this should occur, resuspend the pellet, heat to 65°C for 30 minutes, and repeat step 6.

6. (Step 7) The plasmid DNA should be released from the cell debris and should be found in the supernatant. The viscous material is probably cellular DNA. Minimizing its contamination is desirable.

7. (Step 8) Most CsCl is wet, and the exact weight of undried CsCl will have to be determined empirically.

8. (Step 9) DNA will come to its equilibrium density in about 48 hours; however, RNA will take longer to reach its equilibrium density. The longer the centrifugation, the less RNA contaminating the DNA.

9. (Step 10) Long-wavelength UV is used because short-wavelength UV will cause thymine dimers and cross-links in the DNA.

13. Dissolve precipitated DNA in 50 µl of:

10 mM Tris (pH 7.5)
1 mM Na_3 EDTA
10 µg/ml of RNase A

The amount of DNA obtained is generally sufficient for two gel tracks. The supercoiled plasmid DNA from step 13 can be used directly for gel electrophoresis. All of the steps in this protocol can be conducted at room temperature, except step 7. This step requires long incubation at 0°C and long centrifugation to sediment all cell debris and K-dodecyl sulfate-protein ppt.

Discussion

1. (Steps 1 and 2) Unpurified agar is a potent inhibitor of most restriction endonucleases and other DNA enzymes. The inhibitor may be sulfonated carbohydrates. Picking colonies from regular agar plates can sometimes result in transfer of sufficient inhibitors to cause difficulties with enzymology of the isolated DNA. The use of plates prepared from agarose circumvents these possible difficulties. The entire colony does not need to be used; about one quarter of a colony is usually sufficient.

2. (Step 3) The diethyloxydiformate is used to inactivate nucleases. If biological activity of the DNA is required, this step should be omitted.

3. (Step 4) The SDS lyses the spheroplast and denatures proteins.

4. (Step 5) The heating releases large bacterial DNA from other large cellular components so that it does not pellet in step 8.

5. (Step 6) The KAc precipitates the dodecyl sulfate (DS) and DS-protein complexes. It should be added before cooling the tubes so that the precipitation is slow.

6. (Step 7) Cooling the tubes and waiting 30 minutes assures complete precipitation of the K-DS.

7. (Step 8) If the K-DS precipitate is formed too quickly, it may be flocculant or it may entrap air. As a result it may not sediment easily. The 15-minute sedimentation assures complete removal of K-DS. If a precipitate is still present, transfer to a new tube, mix vigorously, and repeat step 8.

8. (Step 9) Do not remove any precipitate. If the supernatant appears cloudy, resediment.

9. (Steps 10 and 11) The sample contains DNA and RNA and is precipitated easily with ethanol. The sample should not be cooled because undesirable contaminants also precipitate at lower temperatures.

10. (Step 12) The KAc is very soluble in ethanol, so no salt precipitation will occur. All the liquid should be drained off to eliminate the soluble contaminants and the KAc. The tubes can be rinsed with 70% ethanol to aid in elimination of this problem. If the tubes fail to drain, they can be swabbed with a tissue or cotton-tipped applicator, but do not touch the DNA pellet.

11. (Step 13) The RNase is used to remove contaminating RNA that can inhibit DNA enzymes. The digested RNA need not be removed.

12. (Step 14) Sometimes the DNA isolated by this procedure cannot be cleaved with restriction endonucleases. If this is the case, it can be purified further by a second ethanol precipitation. Add 200 µl of 10 mM Tris (pH 7.5), 1 mM Na_3 EDTA, and 50 µl of 5 M KAc. If there is any precipitate, sediment and transfer supernatant to new tube. Add 0.5 ml of ethanol and cool in ice for 20 minutes. Since the RNA has been removed, the DNA is more difficult to precipitate. Repeat steps 11, 12, and 13.

B. Rapid Plasmid DNA Isolation for 10-ml Culture

1. Grow cells to saturation in 10 ml of LB broth plus 0.4% glucose and selective drug, if applicable.

2. Sediment cells in culture tube or 10-ml centrifuge tube and resuspend pellet in 1.4 ml of 10 mM Tris (pH 8.5) and 1 mM Na_3 EDTA. TE

3. Put into a 1.5-ml microfuge tube and centrifuge for 30 seconds.

4. Resuspend in 0.4 ml of 15% sucrose, 50 mM Tris-HCl, and 50 mM Na_2 EDTA (pH 8.5). Mix thoroughly with a Vortex mixer. TES

5. Cool on ice and add 0.1 ml of a freshly prepared 5 mg/ml lysozyme solution in the above buffer at 0°C.

6. Invert gently and occasionally for 10 minutes on ice.

7. Add 0.3 ml of 0.1% Triton X-100, 50 mM Tris-HCl, and 50 mM Na_2 EDTA (pH 8.5) at 0°C. TRT

8. Invert gently and occasionally for 10 minutes on ice.

9. Centrifuge 2 minutes in microfuge located in cold room.

10. Decant supernatant into new microfuge tube.

11. Add 2 µl of diethyloxydiformate and agitate briefly.

12. Heat to 70°C for 15 minutes, cool 15 minutes on ice, and centrifuge 2 minutes in microfuge.

13. Decant supernatant into new microfuge tube.

14. Fill tube with EtOH at room temperature.

15. Sediment precipitate in a microfuge for 5 minutes.

16. Discard supernatant and invert tube on paper towel to drain. All liquid should either drain off or evaporate, but do not overdry.

17. Dissolve precipitated DNA in 50 µl of:

    10 mM Tris (pH 7.5)
    1 mM Na_3 EDTA
    10 µg/ml of RNase A

    5 µl is sufficient for one agarose gel track. The DNA may be cleaved with most restriction enzymes.

PROCEDURE 13

REMOVAL OF ETHIDIUM BROMIDE FROM DNA IN CsCl

Isopropanol or Butanol Extraction

1. Add 1 volume unit of DNA in buoyant CsCl and ethidium bromide to a tube that has a capacity of at least 10 volume units and can be tightly capped and centrifuged (polypropylene or glass are satisfactory, but not polycarbonate).

2. Add 1 volume unit of isopropanol or butanol saturated with aqueous 5 M NaCl, 10 mM Tris, and 1 mM Na_3 EDTA (pH 8.5).

3. Seal tube and mix. Gloves should be worn.

4. Repeat extraction until all visible color is removed. Extract once more. The number of extractions required is greater with more concentrated DNA.

5. Add 2 volume units of H_2O.

6. Add 6 volume units of ethanol and place at -20°C for from 1 hour to several days.

7. Sediment precipitate in centrifuge.

8. Wash pellet with 70% ethanol.

9. Drain and dry pellet.

10. Dissolve pellet in 100 µl of 10 mM Tris and 1 mM Na_3 EDTA (pH 7.5).

Discussion

1. Ethidium bromide is a mutagen (30 μg is as mutagenic as the smoke from one cigarette). Use a tube that will not leak with liquids that have a low surface tension.

2. The high salt saturation of the alcohol assures a two-phase system and that water will not be removed from the aqueous phase.

3. (Steps 3 and 4) The partitioning of ethidium bromide between the two phases is rapid and does not require extensive mixing.

4. (Step 5) Water is added so that the CsCl will not precipitate with addition of ethanol.

5. (Step 6) Large amounts of DNA (50 μg) are readily precipitated by ethanol and do not require extensive cooling. However, since the DNA is very pure, prolonged precipitations are not deleterious.

PROCEDURE 14

λgt HYBRID FORMATION

I. DNA Cleavage

1. Cleave vector and insert DNAs separately. Use of about 5 μg of each should give about 5×10^4 hybrids by transfection.

   DNA buffer = 50 μg/ml or higher DNA
   low-, medium-, or high-salt
   restriction buffer (see Appendix 7)
   10^{-4} M Na_2 EDTA

2. Add 1/10 volume of 0.1 M $MgSO_4$ to give 0.01 M $MgSO_4$.

3. Add endonuclease and incubate at 37°C for 10 minutes. Empirically determine by gel electrophoresis the amount of enzyme to give complete digestion.

4. Repeat step 3.

5. Heat to 70°C for 3 minutes to inactivate endonuclease (see Appendix 7).

6. If DNA is to be packaged in vitro, incubate at 50°C for 15 minutes. If DNA is to be transfected, cool quickly to 0°C.

II. Covalent Joining by T4 DNA Ligase

1. Mix vector and insert DNA, using an equal mass of each.

2. Add ATP to give a final concentration of 1 mM.

3. Add DTT to give a final concentration of 10 mM.

4. Add 0.1 unit of T4 ligase per 1 μg of DNA or 0.1 μl of 1 mg/ml ligase.

5. Incubate at 0–10°C for 1–6 hours.

6. Check covalent joining in the electron microscope or on gels.

7. Infect or package DNA.

References

Thomas, M., J.R. Cameron, and R.W. Davis. 1974. Viable molecular hybrids of bacteriophage lambda and eukaryotic DNA. Proc. Natl. Acad. Sci. 71:4579.

Discussion

The vector and insert DNAs are cleaved separately because it is essential to obtain cleavage of the vector DNA to about one uncleaved λ DNA molecule per 10^4 cleaved λ DNA molecules. λ DNA can be easily prepared with no detectable endonuclease inhibitors. However, many cellular DNA preparations contain inhibitors that, if uncleaved with the λ DNA, will result in a higher concentration of uncleaved λ DNA than 10^{-4}. More endonuclease than empirically determined in step 3 is used to assure complete cleavage. This step is essential only for λ vector DNA. The presence of uncleaved λ DNA at 10^{-4} cannot be readily detected by gel electrophoresis but is easily identified by infectivity. The λ DNA cohesive ends must be joined for the most efficient in vitro packaging but must be left free for the most efficient transfection. The λ cohesive ends have a high activation energy of joining, and therefore a high temperature (50°C) is required to bring about joining effectively. At 0°C, the λ cohesive ends join very slowly. Therefore, restriction-endonuclease-generated cohesive ends can be completely covalently joined without significant λ DNA cohesive end joining. (Section II) The rate of T4 DNA ligase joining is directly proportional to ligase concentration. Therefore, large amounts of ligase generally are used. At the concentrations of ligase recommended, the reaction is completed in a few minutes. The recommended 1–6 hours is excessive time but it is not deleterious.

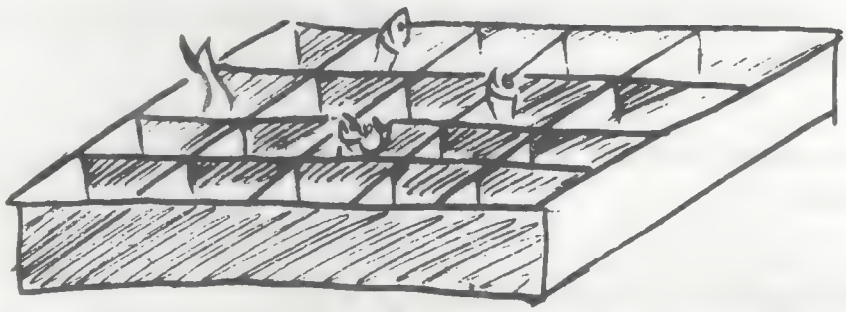
PROCEDURE 15

PACKAGING λ DNA INTO VIRAL PARTICLES IN VITRO

I. Packaging Reaction

1. Use 20 µl of induced packaging cells stored at -70°C.
2. Transfer to ice for thawing (do not warm).
3. After thawing, add 2 µl of 50 mM ATP.
4. Add ≤1 µg of DNA in 1–10 µl.
5. Mix carefully (cells should not have lysed).
6. Collect liquid at bottom of tube by a 3-second centrifugation in a microfuge.
7. Incubate at 37°C for 30–60 minutes.
8. Add 0.2 µl of 1 mg/ml of DNase I.
9. If maximal efficiency of packaging is needed, add an additional 20 µl of thawed extract and 2 µl of 50 mM ATP (increases number of phage by a factor of about two). Incubate at 37°C for an additional 30–60 minutes.
10. Add 200 µl of λ dil and store as a phage stock.
11. Plate no more than 10–15 µl of this stock on one 9-cm plate using a nonrestricting and non-supF strain RD104 (C600 hsdR$^-$ hsdM$^+$). Larger amounts of stock will kill the lawn.

POISSON DISTRIBUTION



The Luria-Delbrück variance test is based on the famous Poisson distribution regardless of whether these are bacterial or fish mutants (Genetics 28 [1943] 491–511). Fish are easier to see but harder to distribute, unless you are a bird.

II. Preparation of Induced Packaging Cells

1. Cell strains
   A: N205 recA$^-$ (λimm434 cI-ts b2 red3 Eam4 Sam7).
   B: N205 recA$^-$ (λimm434 cI-ts b2 red3 Dam15 Sam7).

2. Streak each strain for single colonies and incubate at 25–30°C.

3. Check several colonies for growth at 30°C and 42°C.

4. Pick one colony of each strain from the 30°C plate that does not grow at 42°C.

5. For each strain, suspend colony in 200 ml of LB broth (30°C) and grow cells at 30°C to an A_{600} = 0.3.

6. Induce the two cultures by incubating at 42–43°C for 15 minutes.

7. Incubate at 37°C for 3 hours.

8. Test a 1-ml sample from each culture for induction by adding a drop of $CHCl_3$ and incubating at 37°C for 5 minutes. The culture should lyse.

9. Mix the two 200-ml cultures and sediment at 5000 rpm for 10 minutes.

10. Resuspend in 40 ml of cold λ dil and sediment at 5000 rpm for 10 minutes.

11. Resuspend in 10 ml of packaging buffer:

    0.04 M Tris (pH 8)
    0.01 M $MgSO_4$
    0.01 M spermidine-3HCl
    0.01 M putrescine-2HCl
    0.1% β-mercaptoethanol
    7% DMSO

12. Sediment at 5000 rpm for 5 minutes.

13. Resuspend in 1 ml of packaging buffer and distribute in 20-µl samples in 0.5-ml polypropylene centrifuge tubes.

14. Freeze in liquid nitrogen and store at -70°C. Can be stored for several months.

References

Hohn, B. and K. Murray. 1977. Packaging recombinant DNA molecules into bacteriophage particles in vitro. Proc. Natl. Acad. Sci. 74:3259.

Sternberg, N., D. Tiemeier, and L. Enquist. 1977. In vitro packaging of a λDam vector containing EcoRI DNA fragments of E. coli and phage P1. Gene 1:255.

PROCEDURE 16

TRANSFECTION OF λ DNA

I. Cell Growth

1. Start from a colony on a plate. Inoculate 10 ml of LB broth in a 125-ml flask and incubate for 20 hours at 37°C.

2. Dilute bacterial culture from mixture 1 (above) by 1/100 into LB broth. Use 5 ml of broth per transfection (generally, 50 ml of broth in a 500-ml flask). Incubate in an agitator at 37°C until A_{600} = 0.6–0.7. This usually requires 2–3 hours of growth. Add thymidine to 50 μg/ml at A_{600} = 0.3.

II. Cell Preparation

1. Sediment cells at 5000 rpm for 5 minutes at 2°C.

2. Resuspend in CT media (50 mM $CaCl_2$ and 50 μg/ml of thymidine to half the original volume). All solutions, tubes, and pipettes must be at 0°C (ice water).

3. Incubate at 0°C (ice water) for 5 minutes.

4. Resediment cells at 5000 rpm for 5 minutes at 2°C.

5. Resuspend in CT media in 1/20 of the original volume at 0°C.

III. DNA Infection

1. Add 0.2 ml of cells to 0.1 ml of DNA (max 100 ng) in 0.1 M Tris at pH 7.2 (0.09 M Tris-HCl + 0.01 M Tris base) and 50 μg/ml of thymidine at 0°C (in a thin-walled glass tube at 0°C).

2. Incubate 3–60 minutes at 0°C; 2 minutes at 45°C. Plate on room-temperature plates with 2.5 ml of LB soft agar.

3. Efficiency should be at about 2×10^3 plaques/ng of λ DNA or about 1 plaque/10^4 molecules.

IV. Cell Storage

1. Cells treated with Ca^{++} may be frozen in CT plus 7% dimethyl-sulfoxide media by immersion in liquid N_2. 1-ml aliquots are stored in liquid N_2.

2. Cells are thawed at 0°C. Then, follow procedures outlined in step III-1.

3. Alternatively, cells may be resuspended in 0.01 M $MgSO_4$ as in step II-2 and stored at 4°C for about 1 week. Transfection efficiency of λ DNA is generally reduced two- to tenfold.

Discussion

I. The past history of the culture seems to affect its transfection efficiency. Therefore, always grow an overnight culture first, followed by a culture diluted 1/100. The culture should not grow to an A_{600} greater than 0.7. If this should occur, start a fresh culture diluted 1/100. Good aeration is important, so use a large flask. The thymidine is added because it increases the transformation efficiency two- to fourfold with some bacterial strains.

II. Keeping the cells very cold (0°C) is essential for high transformation efficiency. Make certain the cells are not warmed even for a short period of time.

III. More DNA than 100 ng/transfection may reduce the total number of plaques per plate. The thin-walled glass tube (not plastic) is used to achieve a more rapid heat shock. This is very important for λ, but not for plasmids, so make sure the heating to 45°C is achieved quickly. 2×10^3 plaques/ng is the expected efficiency, not the maximum ever achieved.

IV. Transfection efficiency of λ DNA is generally reduced two- to tenfold on freezing the cells. Cells frozen for 9 months have been used successfully.

Cells

SF8 (*E. coli*) *hsdR*$^-$ *hsdM*$^-$ *recB* *recC* *lop*-11 (ligase overproducer) *supE*44 (*su2*$^+$) *gal*-96 Sm^R *leuB*6 *thi*-1 (B1$^-$) *thr*$^-$

HB101 (*E. coli*) *hsdR*$^-$ *hsdM*$^-$ *recA*13 *supE*44 (*su2*$^+$) *lacZ*4 *leuB*6 *proA*2 *thi*-1 (B1$^-$) Sm^R

BNN45 (*E. coli*) *hsdR*$^-$ *hsdM*$^+$ *rec*$^+$ *supE*44 (*su2*$^+$) *supF* (*su3*$^+$) B1$^-$ *met*$^-$

Reference

Mandel, M. and A. Higa. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53:159.

PROCEDURE 17

SUBCLONING DNA FRAGMENTS INTO *E. coli* PLASMID VECTORS

1. Cleave about 250 ng of plasmid DNA (e.g., pBR322) to completion with the appropriate endonuclease(s) in a 1.5-ml microfuge tube.

2. Add 0.1 µl or less of 1 mg/ml of calf intestine alkaline phosphatase.

3. Incubate at 37°C for 15 minutes.

4. Add an equal volume of distilled phenol, mix with a Vortex mixer, and then centrifuge.

5. Remove upper aqueous phase with a polypropylene pipette tip and transfer to a new 1.5-ml microfuge tube.

6. Add about 0.1 ml of buffer-saturated ether, mix with a Vortex mixer, centrifuge, and then discard upper ether phase.

7. Repeat ether extraction twice more.

8. Blow off ether.

9. Cleave DNA to be subcloned (use about 50 ng of insert fragment) with appropriate endonuclease(s).

10. DNA fragment to be subcloned may be isolated by agarose gel electrophoresis (optional; see Procedure 25).

11. Mix equal molar amounts of vector DNA and insert DNA.

12. Add ATP to give a final concentration of 1 mM.

13. Add DTT to give a final concentration of 10 mM.

14. Add 0.1 unit of T4 ligase or 0.1 µl of 1 mg/ml.

15. Incubate at 0–10°C for 30 minutes to 3 days.

16. Transfect RD102 = HB101/λ.

17. 50 ng of phosphatase- and ligase-treated vector DNA alone usually gives 0–10 colonies.

Reference

St. John, T. (pers. comm.).

PROCEDURE 18

TRANSFORMATION WITH PLASMID DNA

1. Dilute overnight LB culture of HB101, RD102 = HB101/λ, or BNN45 1:100 into fresh LB broth (40 ml in 250-ml flask) at 37°C. This amount of culture is sufficient for 20 transformations.

2. Collect cells at A_{600} = 0.6 (2–3 hr) and sediment at 5000 rpm for 5 minutes at 2°C.

3. Resuspend in 20 ml of 50 mM $CaCl_2$ for 5–60 minutes at 0°C.

4. Sediment cells at 5000 rpm for 5 minutes at 2°C and resuspend in 2 ml of 50 mM $CaCl_2$ for 5–60 minutes at 0°C.

5. Add 0.1 ml of cells to DNA in 0.1 ml of 0.1 M Tris (0.09 M Tris-HCl, 0.01 M Tris base) at pH 7.2 for 10 minutes at 0°C.

6. Heat treat: Use 2 minutes at 37°C.
   10 minutes at room temperature, 2 minutes at 37°C plus 10 minutes at room temperature, 2 minutes at 45°C plus 10 minutes at room temperature, and 2 minutes at 45°C are all equal in efficiency (unlike with λ).

7. Add 1 ml of LB broth and incubate at 37°C for 20 minutes.

8. Add 2.5 ml of LB soft agar (with no drugs) at 47°C and pour on LB plates containing 10 μg/ml of tetracycline or 50 μg/ml of ampicillin (fresh). Tetracycline plates can be stored at 10°C for a few weeks before use. Ampicillin plates can be stored at 10°C for a few days before use.

   Efficiency using either HB101 or BNN45 is about 4×10^2 transformants/ng of form-I pBR322 DNA (using 5 ng of DNA/plate).

Saturation occurs under these conditions for both HB101 and BNN45 between 50 ng and 100 ng of DNA per plate.

EcoRI linears of pBR322 are probably not infectious in HB101 (background of 0.3–0.5% may equal uncut molecules), but apparently are infectious in SF8, at 2–3% of closed circular DNA.

Reference

Mandel, M. and A. Higa. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53:159.

PROCEDURE 19

HYBRID PHAGE COMPLEMENTATION IN *E. coli*

I. Lytic Selection from Hybrid Pool

1. Grow auxotrophs to late exponential growth in M9 + 0.2% maltose + nutritional requirements (40 μg/ml of each amino acid). Count cell density.

2. Sediment (8000 rpm, 5 min) and resuspend in 1/10 volume of 10 mM $MgSO_4$ or volume necessary to give 10^{10} cells/ml.

3. Adsorb 2×10^6 phage or less with 2×10^9 bacteria for 15 minutes at 37°C. Plating efficiency of complementing phage is about 1.

4. Plate by adding 2.5 ml of M9 soft agar (~0.6%). Growth of complementing phage may take 6–40 hours of incubation at 37°C.

5. Plaque-purify on nonselecting plate (LB plate) and retest complementation.

Discussion

1. At higher concentrations of bacteria, a sufficient lawn is often present to allow visualization of plaques. Addition of 10 ug of ethidium bromide per plate, visualized under long-wavelength UV light, may also be used. λ growth still depends on complementation of the bacterial lesion at these high densities.

2. Up to 10^7 phage have been plated without obscuring low-frequency complementing signals. The plating efficiency is 1.

3. If necessary, replicas can be made by using sterile Millipore filters. Positive signals will produce large areas of lysis on a wild-type E. coli lawn after a short incubation at 37°C.

4. Leaky bacterial mutants often allow noncomplementing phage to grow after long periods of incubation. Complementing viruses can sometimes still be distinguished by large plaque sizes or early appearance.

5. Beware of phage growth around revertants.

6. Complementation may or may not depend on the orientation of the gene. E. coli hisB mutants can only be complemented by λ-yeast hybrids if the yeast his-3 sequence is oriented in the same direction as p_L. However, RD105 ([E. coli] trpC9830) can be complemented by yeast sequences in either orientation. Furthermore, trpC complementation can occur in the complete absence of p_L. Differences in the directional specificity may be due either to the strength of the selection--λ lytic growth requires very little tryptophan biosynthesis--or to the efficiency of expression of the eukaryotic sequence.

Reference

Stinchcomb, D. (pers. comm.).

II. Double-lysogen Selection from Hybrid Pool

A. Selection

1. Prepare high-titer plate stocks of hybrid phage pool and of integration helper phage (λgt4-lac5) on a cell that will not grow on selective minimal media (BNN45).

2. Grow auxotrophs to late exponential growth in 50 ml of M9 + 0.2% maltose + nutritional requirements (40 μg/ml of each amino acid). Count cell density.

3. Sediment (8000 rpm, 5 min) and resuspend in 1/10 volume of 10 mM $MgSO_4$ or volume necessary to give 10^{10} cells/ml.

4. Mix 5×10^8 cells with 2×10^9 helper phage and 10^8 hybrid phage.

5. Incubate at room temperature for 15 minutes.

6. Mix with 2.5 ml of M9 minimal soft agar and pour on minimal plate without selecting nutrient.

7. Grow at 32°C, because the integration helper carries the cI857 temperature-sensitive repressor lesion (requires 1–3 days).

8. Control plates. (1) Cells with no phage; (2) phage with no cells; (3) use less hybrid phage pool (10^7 or 10^6 phage).

9. Pick each resulting colony and suspend in 0.1–1.0 ml of LB broth.

10. Incubate at 32°C until there is a slight turbidity (10^7–10^8 cells/ml); then incubate at 42°C for at least 20 minutes.

Continue incubation at 37°C for 2 hours. Add one drop of $CHCl_3$ to complete lysis.

11. Streak resulting lysate for single plaques on an LB plate with BNN45 host. Add 40 µl of 40 mg/ml of Xgal to the soft agar to identify the λgt4-lac5 integration helper (blue plaques).

12. Prepare high-titer plating stocks from nonblue plaques.

13. Retest complementation starting at step 4.

B. Materials

1. Hybrid phage: any att^-

   Helper phage: any att^+int^+ phage with a functional homologous immunity, e.g., λgt4-lac5.

2. Cell: any auxotroph that will plate λ and can be grown on minimal media plus nutritional requirements.

3. Plates: M9

Discussion

Most of the λ cloning vectors do not contain a functional attachment site (*att*$^+$) or integration gene (*int*$^+$) and thus cannot form stable lysogens. However, a helper phage can be used that will integrate. The λ hybrid can then integrate via homologous λ recombination. These double lysogens form at a frequency of about 1%. They cure rather easily, but can be maintained with selection.

1. The suggested helper is λgt4-*lac*5. It carries the λ*c*I857 repressor gene and the *E. coli* β-galactosidase gene (*lacZ*). The plating stocks are prepared using a host that will not grow on minimal media. This is to eliminate the possibility of cells contaminating the phage stocks that can grow on the selecting plates.

2. Growth on minimal media is to adapt the cells to growth on this media. The maltose is used to assure good adsorption of λ.

3. If the cell density is much less than 10^9 cells/ml before sedimentation, they can be resuspended in a smaller volume to give a density of about 10^{10} cells/ml.

4. These ratios of cells and phage assure that most cells will be multiply infected with the helper and that few cells will be infected by more than one hybrid. Note that a lot of cells are being used on each plate. If the mutation reverts at 10^{-7}, then several colonies will appear on the minimal plates as revertants. These revertants are likely to contain an integrated λ helper and may contain a random λ hybrid. Therefore, retesting the complementation is essential.

5. Incubation at high cell and phage density assures good adsorption.

6. The soft agar is made from agar dissolved in water. It can be melted just before use, and minimal media can be added after it has cooled to 50°C.

7. Most integration helpers will contain cI857, a temperature-sensitive repressor lesion. Therefore, the temperature should never get above 37°C. The use of cI857 aids recovery of the hybrid in step 10.

8. The control plates are essential for a quick assay of the likelihood of a complementation event. Cells with no phage measure the number of revertants in the cell preparation. Each preparation may have a different number of revertants. The phage with no cells measure the number of cells that can grow on the minimal plates in the phage preparation. Using different numbers of hybrid phage allows a quick assessment, since the number of true complementation events should be directly proportional to the number of hybrid phage. Typically, a hybrid pool from Salmonella gives about ten complementing colonies per plate.

9. (Steps 9 and 10) Incubation of the cell suspension at 42°C induces the lysogens (if they contain cI857). The induction is irreversible after 20 minutes.

10. (Steps 11 and 12) The integration helper contains lac and will give blue plaques on plates containing Xgal. The nonblue plaques should be hybrids and may be responsible for the cell growth.

11. (Step 13) A repeat of the complementation is essential to confirm the effect.

Reference

Struhl, K., J.R. Cameron, and R.W. Davis. 1976. Functional genetic expression of eukaryotic DNA in Escherichia coli. Proc. Natl. Acad. Sci. 73:1471.

PROCEDURE 20

AGAROSE GEL ELECTROPHORESIS

I. Agarose Gel

Agarose gel electrophoresis is conducted in a horizontal configuration, because (1) it provides better support at low agarose concentration, (2) there is less distortion (collapse) during electrophoresis, and (3) the bands of DNA are less distorted. The easiest gel system to operate seems to be one that has the gel completely submerged by about 1 mm in the electrophoresis buffer. The resistance of the agarose is not much greater than the buffer, and a large fraction of the current is carried in the agarose.

A. Buffers

All neutral pH buffers may contain 0.5 μg/ml of ethidium bromide.

1. Tris-borate

	10× buffer/liter
89 mM Tris OH	108 g
89 mM boric acid	55 g
2.5 mM Na_2 EDTA	9.3 g
pH 8.3	

2. Tris-phosphate

	10× buffer/liter
89 mM Tris OH	108 g
23 mM H_3PO_4	15.5 ml of 85% H_3PO_4 (1.679 g/ml)
2.5 mM Na_2 EDTA	9.3 g
pH 8.3	

3. Tris-acetate

	50× buffer/liter
40 mM Tris OH	242 g
20 mM acetic acid	57.1 ml of glacial acetic acid
2 mM Na_2 EDTA	37.2 g
pH 8.1	

4. Alkaline	100× buffer/liter
30 mM NaOH	166 ml of 50% NaOH
2 mM Na_2 EDTA	74.5 g

During electrophoresis, the anode becomes alkaline and the cathode becomes acidic. Therefore, a high-capacity buffer system is usually used. No Cl^- is present because it has no buffer capacity and it can lead to loss of biological activity of the DNA in the gel.

1. Tris-borate. A high-capacity buffer electrolyte that probably gives the sharpest bands. No microorganisms will grow in the 10× stock, but an alkaline-soluble precipitate forms on long storage. Agarose gels in the presence of borate cannot be dissolved by a high concentration of $NaClO_4$ or KI.

2. Tris-phosphate. Also a high-capacity buffer that gives results similar to Tris-borate. However, microorganisms might grow in the stock solution. It has the advantage that the gels can be dissolved in concentrated $NaClO_4$ or KI.

3. Tris-acetate. The buffer capacity of this system is rather low, and long electrophoresis may require recirculation of the buffer tanks. High-voltage gradients can be used without heating. Microorganisms might grow in the stock solutions. Gels in Tris-acetate can be dissolved in concentrated $NaClO_4$ or KI. This is probably the most frequently used buffer.

4. Alkaline. This buffer has very low capacity and, generally, recirculation of the buffer tanks is required. Samples cannot be loaded with Mg^{++} present or the DNA will precipitate. The Mg^{++} must be chelated with excess EDTA.

4. Alkaline	100× buffer/liter
30 mM NaOH	166 ml of 50% NaOH
2 mM Na_2 EDTA	74.5 g

During electrophoresis, the anode becomes alkaline and the cathode becomes acidic. Therefore, a high-capacity buffer system is usually used. No Cl^- is present because it has no buffer capacity and it can lead to loss of biological activity of the DNA in the gel.

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4. Alkaline. This buffer has very low capacity and, generally, recirculation of the buffer tanks is required. Samples cannot be loaded with Mg^{++} present or the DNA will precipitate. The Mg^{++} must be chelated with excess EDTA.

B. Agarose

There is considerable variability with different sources of agarose with respect to the solidity, resolution of DNA fragments, electrophoretic mobility of DNA, ease of melting, transparency, and interfering contaminants. The main contaminant is probably sulfonated agaroses, which inhibit many nucleic acid enzymes.

Generally, we have used agarose from MC/B. The agarose is dissolved in electrophoresis buffer by bringing to a boil in a microwave oven. Be sure the solution is completely homogeneous and no solid particles of agarose remain. The solution is cooled to 50°C before pouring the gel. Pouring the gel too hot can easily warp the electrophoresis apparatus.

C. Sample Wells

The sample wells are made with a comb made of lucite, PVC, or teflon, inserted into the molten gel. The comb is adjusted before pouring the gel so that the bottom of the teeth are about 0.5 mm off the gel bed. If the wells break through to the bottom, the sample may leak underneath the gel. After the gel is completely set, the comb is removed and the wells are filled with electrophoresis buffer.

D. Loading Samples and Tracking Dye

Samples are prepared containing 5–10% glycerol or 5–10% sucrose and 0.025% of a tracking dye. For example, a 1/10 volume of a solution containing 50% glycerol and 0.25% dye is added to the sample.

DNA. Load about 10 ng of DNA for each expected band. The gel will become overloaded if more than about 100 ng of DNA is in a band.

Tracking Dye

bromphenol blue (decomposes in alkali)
bromcresol green (same mobility in neutral and alkali)
xylene cyanole FF (decomposes in alkali, lower mobility than BPB or BCG)

Samples can be loaded with a pipetter and polypropylene tip or a microsyringe with small-gauge plastic tubing.

Small DNA molecules can have the same or greater mobility as the tracking dye. The lower the agarose concentration and/or the higher the voltage gradient, the larger the DNA fragment that will have the same electrophoretic mobility as the tracking dye. The tracking dye will adsorb the fluorescence from DNA-bound ethidium bromide, resulting in failure to observe faint DNA bands in the tracking dye. Samples can be loaded without tracking dye.

E. Voltage Gradient

Voltage gradients normally employed are between 0.5 and 5 V/cm. Higher resolution, especially for high-molecular-weight DNA (>70 kb), is achieved at low-voltage gradients. Higher voltage gradients are used on small DNA (<2 kb) to increase the mobility, thus reducing the total amount of diffusion of the DNA band. The most frequently used system is 0.7% agarose at 1 V/cm for about 12 hours with Tris-acetate.

The mobility of DNA is almost inversely proportional to the log of the molecular length.

Duplex length	Agarose (%)	Voltage gradient (V/cm)
150–1000	1.8	2–3
300–2500	1.4	2–3
500–4000	1.0	1–2
700–6000	0.7	0.5–1
1000–9000	0.5	0.5–1

In Tris-acetate buffer, the tracking dye will electrophorese at about 1 cm/hr using 1 V/cm.

References

McDonell, M.W., M.N. Simon, and F.W. Studier. 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. 110:119.

Peacock, A.C. and C.W. Dingman. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. Biochemistry 7: 668.

II. Staining DNA in Agarose Gels for Nucleic Acid

Caution: Ethidium bromide or its metabolized product and acridine orange are mutagens. In the Ames test, 90 μg of ethidium bromide and 80 μg of acridine orange are each equal to the smoke condensate from one cigarette or 2 μg of nitrosoguanidine. Therefore, wear gloves while handling these solutions.

A. Ethidium Bromide

Ethidium bromide binds to double-stranded nucleic acid by intercalation. Ultraviolet irradiation adsorbed by the ethidium bromide (300 nm) or by the DNA (260 nm) and energy transferred to the ethidium bromide can be re-emitted as fluorescence at 590 nm. Best staining can be achieved at about 0.5 μg/ml by incorporation into the gel and electrophoresis buffer before electrophoresing the nucleic acid. The gel can be stained after the run by soaking it in 0.5 μg/ml of ethidium bromide in buffer for about 1 hour. Destaining in either case is not necessary. Single-stranded nucleic acid can also be stained by soaking the gel in 5 μg/ml of ethidium bromide and 1 mM $MgSO_4$ for 1 hour and then destaining for 1 hour in 1 mM $MgSO_4$.

Commercially available ethidium bromide is very impure (only about half the weight is ethidium bromide). Its concentration can be determined spectrophotometrically.

A_{480} = 5700 M^{-1} cm^{-1} and molecular mass = 394 g/mole

B. Acridine Orange

Acridine orange can bind to double-stranded nucleic acid by intercalation or can bind single- and double-stranded nucleic acid by electrostatic interaction with the phosphate. Ultraviolet irradiation adsorbed at 260 nm can be fluoresced by double-stranded nucleic acid at 530 nm (green) or by single-stranded nucleic acid at 640 nm (red).

Staining of gels is at 30 μg/ml of acridine orange for 1 hour in 10 mM salt and destaining for 1 hour in 0.1 mM salt.

III. Photography of Gels Containing Nucleic Acids

Many dyes that bind to nucleic acids will fluoresce when irradiated with UV light. Some dyes, such as ethidium bromide, have a planar group that is free to rotate in solution. However, when bound to DNA, this rotation may be restricted. As a result of such restriction, the efficiency of fluorescence is much greater for these dyes when bound to nucleic acids than in nonviscous solutions. UV light can be adsorbed by nucleic acids, the energy transferred to dyes, and then fluoresced.

Generally, the most sensitive photograph can be achieved by irradiation at about 260 nm. However, such irradiation rapidly destroys the viability of nucleic acids (λ DNA infectivity decreases one log for every 1-sec exposure). A few minutes of exposure at 300 nm does not cause detectable loss of infectivity of native λ DNA (with or without ethidium bromide), but does cause some detectable cross-linking.

A. UV Light Source

1. 254-nm transilluminator (C61, UV Products)

2. 300-nm transilluminator (C62, UV Products)

B. Filters

1. UV (J-344 contrast filter, UV Products)

2. Ethidium bromide at 590 nm – Kodak Wratten gelatin filter 23A.

3. Acridine orange for 530 nm and 640 nm – Kodak Wratten gelatin filter 12 for both green (double-stranded) and red (single-stranded), or 40 for green only and 29 for red only.

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C. Camera (Polaroid MP-4 Land camera).

D. Film (Polaroid 667 or 665 positive/negative).

Since most filters fluoresce when irradiated with UV light, the UV contrast filter is always placed between the colored filters and the UV light source. The 254 UV transmitting filter on the C61 transilluminator becomes UV opaque on prolonged exposure. The half-life is about 20 hours. The UV contrast and gelatin filters also probably deteriorate with prolonged exposure and should be replaced periodically. The 230–270-nm intensity of the C61 transilluminator at the surface with a new filter is 2400 $\mu W/cm^2$ and without a filter is 8500 $\mu W/cm^2$.

IV. Glyoxal Gels

A. Sample Preparation

1. Either ethanol-precipitate nucleic acid samples and resuspend in H_2O or, if they are concentrated, proceed directly.

2. 50-µl sample in microfuge tube:

   25 µl of DMSO (may be decreased or omitted if more sample volume is needed)
   2.5 µl of 0.2 M $NaPO_4$ (pH 7.0)
   7.1 µl of 7 M deionized glyoxal
   15.3 µl of sample

3. Cap tube and incubate at 50°C for 60 minutes.

4. Add sucrose or glycerol and BPB dye marker and load sample into gel.

B. Gel

1. Electrophoresis buffer is 10 mM $NaPO_4$ (pH 7.0).

2. Gel is 1–1.5% agarose or 0.5% agarose and 1.5–2.5% polyacrylamide.

3. Electrophorese 4–20 hours at 1 V/cm.

4. Buffer tanks must be recirculated.

5. a. Stain with 30 µg/ml of acridine orange for 30 minutes. Destain by soaking the gel in 1 mM Na_3 EDTA for 1 hour.

   or

b. Reverse the glyoxalation to prevent its blockage of nucleic acid hybridization by soaking the gel in 200 ml of 50 mM NaOH for 30 minutes. Neutralize the gel and stain the nucleic acid by soaking the gel in 200 ml of 0.2 M NaAc (pH 4.0) and 1 mg/ml of ethidium bromide for 30 minutes.

Destain by soaking the gel in 0.2 M NaAc (pH 4.0) for 30 minutes.

6. Photography

a. Ethidium bromide: Irradiate at 254 nm and photograph with UV filter and orange filter.

b. Acridine orange: Irradiate at 254 nm and photograph with UV filter and yellow filter.

Double helical nucleic acid - green fluorescence at 530 nm.

Single-stranded nucleic acid - red fluorescence at 640 nm.

C. Glyoxal Preparation

1. Glyoxal (technical grade) 30% in H_2O ~7 M. It is readily oxidized and the oxidation products can be removed by ion-exchange chromatography. Among the oxidation products are glycolic acid, glyoxylic acid, and formic acid.

2. Deionize by passing through an AG-501-X8 or AG-501-X8(0) column (10-ml pipette) (0.5 ml of resin per 1 ml glyoxal).

3. Store in sealed tubes (tightly capped with a small air space) at -20°C.

D. Glyoxal: Adduct Reaction and Stability

pH <6 - stable

pH 7 - stable at least 20 hr

Decomposition rate constants: pH 8.0 .001 min^{-1}

pH 10 .02 min^{-1}

pH 11 .06 min^{-1}

References

McMaster, G.K. and G.G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. 74:4835.

St. John, T. (pers. comm.)

PROCEDURE 21

TRANSFER OF DNA TO NITROCELLULOSE OR DIAZOTIZED PAPER

I. Transfer from Agarose Gels

A. Agarose Gel

1. Electrophoretically separate DNAs using a horizontal 14.5 × 13.5 × 0.8-cm (150 ml) gel electrophoresis apparatus. We usually use 0.7% agarose and Tris-acetate buffer.

2. Each 0.4-cm slot can be loaded with up to 5 μg of restriction-endonuclease-cleaved DNA. We use about 1 μg of cleaved bacterial DNA.

3. Ethidium bromide (0.5 μg/ml) is included in the electrophoresis buffer, and the gel is photographed under short-wave UV light.

B. Breakage of Large DNA in Gel by Depurination

These depurination steps are not essential if the DNA is prepared by the rapid isolation method. Apparently, it is sufficiently nicked by the diethyloxydiformate and heat treatment to allow rapid and complete transfer. One may omit steps 1–3 (below) and start with step C-1.

1. Place the gel (14.5 × 13.5 × 0.8 cm [150 ml]) in a tray and add 250 ml of 0.25 M HCl at room temperature.

2. Rock occasionally for 15 minutes, decant the acid, and repeat steps 1 and 2.

3. Rinse with water briefly and proceed immediately to step C.

C. Alkaline Denaturation

1. Add 250 ml of 0.5 M NaOH and 1.5 M NaCl and gently agitate for 15 minutes.

2. Decant alkali and repeat step C-1.

D. Neutralization

1. For transfer to nitrocellulose, neutralize with 500 ml of 0.5 M Tris-HCl (pH 7.5) (60 g Tris base and 30-ml concentration of HCl per liter) and 1.5 M NaCl (90 g/liter) at room temperature for 30 minutes with gentle agitation.

2. For transfer to diazotized paper, neutralize with two 250-ml portions of 1 M NaAc (pH 4.0) for 30 minutes each. Remove the high salt by soaking the gel in 0.1 M NaAc (pH 4.0) for 30–60 minutes.

E. Transfer to Paper

1. Prepare a stack of 12 sheets of Whatman 3MM paper (57 × 46 cm, cut or folded and torn into quarter sheets) on a sheet of plastic wrap or in a tray and saturate with buffer. Two sheets are wet and laid down at a time; the bubbles are rolled out with a rod or pipette. Buffer: Use 20× SSPE (see Procedure 23) for transfer to nitrocellulose and 0.1 M NaAc (pH 4.0) for transfer to diazotized paper.

2. Place the gel on the 3MM paper.

3. A nitrocellulose filter sheet (S&S B85 or HAWP Millipore), or a diazotized filter sheet cut to the size of the gel, is wet with water and placed on top of the gel without air bubbles between the gel and filter.

4. Five sheets of 3MM paper (the same size as the filter) wet with water are placed on top of the nitrocellulose filter without air bubbles between the sheets.

5. A stack of about 6 cm of paper towels cut to size are placed on top of the 3MM paper. The stack is uniformly compressed with a 1-kg weight on a thick sheet of plexiglass.

6. Allow the transfer to occur for 2 hours or longer.

7. The filter is turned over with the shrunken gel attached, and the gel lanes and edges are marked with a soft-lead pencil.

8. The nitrocellulose filter is rinsed in 2× SSPE for 10 minutes and dried in a vacuum oven at 80°C for 2 hours.

9. Hybridization is conducted as described in Procedure 23.

References

Loh, E.Y. (pers. comm.).

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503.

Wahl, G.M., M. Stern, and G.R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. 76: 3683.

II. Transfer from Phage λ Plaques

1. Grow λ plaques on LB plates with 10^2–10^5 plaques. Use dry or 2-day-old plates.

2. Cool resulting plaque plates at 4°C for about 15 minutes to several hours to harden agar.

3. Place an 82-mm dry nitrocellulose filter (S&S BA85 or HAWP Millipore) on the lawn of cells, with no air bubbles being allowed to form between the agar and the filter.

4. Adsorb for 1–20 minutes.

5. a. During adsorption, mark the filter and plate for orientation. One method is to stab three spots at the edge of each filter (while on the plate) with a 25-gauge syringe needle containing waterproof black drawing ink mixed with 1 μg/ml of denatured, unlabeled probe DNA. Apply about 10 μl at each spot. Mark each plate uniquely and nonsymmetrically.

   b. An alternate method for filter orientation is to incorporate into the plaque plate a phage that can be uniquely identified on the plate and that will also hybridize to the probe. Spot about 10 λgt5-lac5,pBR322 phage on the lawn before growth or add 10–20 λgt5-lac5,pBR322 phage to the 10^2–10^5 phage to be screened. Plate with top agar containing Xgal (40 μl of 40 mg/ml in DMSO stored at -20°C).

6. Carefully remove filter from plate.

7. Dip filter or batch wash in 0.5 M NaOH and 1.5 M NaCl for 20 seconds to 5 minutes.

8. Dip filter or batch wash in 0.5 M Tris (pH 7.5) and 1.5 M NaCl for 20 seconds to 5 minutes.

9. Rinse in 2× SSPE (see Procedure 23).

10. Blot and bake at 80°C in a vacuum for 1 1/2–2 hours.

11. Hybridize as in Procedure 23.

12. After developing the X-ray film, align it with the filters in plastic wrap and mark the film at the orientation and identification marks on the filters.

13. Align original plate with marked X-ray film for identification of hybridizing plaque.

7. (Steps 7–10) These are standard procedures for preparation of nitrocellulose. The exact operations are not critical, since weakened signals are still obtained upon omission of steps 7, 8, and 9. Do not bake the filters for longer than 2 hours or at heat higher than 80°C because the filters will become even more brittle than normal.

8. (Steps 11 and 12) The alignment of the exposed spots on the X-ray film with the plaque plate is usually difficult. We generally pick several plaques in the area of a positive signal with a toothpick or platinum wire and stab them onto a lawn of sensitive cells. A large plaque can be obtained by stabbing five times in a small area. Use a numbered grid pattern. Use this grid of plaques to make a new filter. After the correct phage spot is identified, it is plaque-purified and retested.

Reference

Benton, W.D. and R.W. Davis. 1977. Screening of λgt recombinant clones by hybridization to single plaques in situ. Science 196:180.

III. Transfer from Bacterial Colonies

1. Grow colonies on a selecting plate in a grid pattern.

2. Prepare a filter replica on dry nitrocellulose (82 mm; S&S BA85 or HAWP Millipore) or moist (with broth) Whatman 540 paper by one of the following methods:

   a. Drop the filter directly on the colonies while they are quite small and transfer to a fresh selecting plate with the cell side away from the media and toward the air. Only one replica can be prepared.

   b. Prepare a velvet replica and replica plate to the filter previously applied to a selecting plate. More than one replica can be prepared.

   c. Grow colonies in a microtiter well and transfer to the filter with prongs in a holder. Many replicas that have the same number of cells can be prepared.

3. After the colonies have grown on the filter, carefully remove the filter and mark it for orientation. Use a soft-lead pencil or cuts in the filter. Also, application of 10 ng of denatured, unlabeled probe DNA in a distinctive pattern will aid in orienting the X-ray film later and will test the hybridizability and labeling efficiency of the labeled probe DNA. Each filter should be marked uniquely and nonsymmetrically.

4. Place filter, colony side up, on two sheets of 3 MM paper (14 × 23 cm) containing 30 ml of 0.5 M NaOH and 1.5 M NaCl for 3–10 minutes.

5. Transfer to a piece of dry 3 MM paper, colony side up, to remove excess liquid.

6. Place filter, colony side up, again on two sheets of 3 MM paper (14 × 23 cm) containing 30 ml of 0.5 M Tris (pH 7.5) and 1.5 M NaCl for 3–10 minutes.

7. Place filter, colony side up, on a suction apparatus and place under strong vacuum. Rinse twice with 25 ml of 90% ethanol and aspirate until they are dry.

8. Blot between filter paper and bake with filters pressed at 80°C in a vacuum for 1 1/2–2 hours.

9. Hybridize as in Procedure 23.

10. After developing the X-ray film, align it with the filters in plastic wrap and mark the film at the orientation and identification marks on the filter.

11. Align original plate with marked X-ray film for identification of hybridizing colony.

Reference

Grunstein, M. and D.S. Hogness. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. 72:3961.

PROCEDURE 22

α-^{32}P-LABELING OF DNA BY NICK TRANSLATION

Reaction is given for 25 µl.

Nick Translation Buffer

50 mM Tris-HCl (pH 7.5)
10 mM $MgSO_4$
1 mM DTT
50 µg/ml of BSA

I. Reaction

1. H_2O; volume = 20 µl - (volume DNA solution added to microfuge tube).

2. 2.5 µl of 10× NT reaction buffer:

   0.5 M Tris (pH 7.5)
   0.1 M $MgSO_4$
   10 mM DTT
   500 µg/ml of BSA

3. 2.5 µl of a solution containing 0.2 mM each dNTP minus dNTP containing ^{32}P.

4. 0.5 µg or less of DNA.

5. 0.5 µl of diluted DNase (see III).

6. Transfer above reaction mixture to ^{32}P-labeled dNTP in microfuge tube (see II).

7. 0.1 µl of 2 mg/ml of homogeneous E. coli DNA polymerase I.

8. Incubate at 14°C for about 3 hours.
   Incorporation will generally show the following sequence: a delay, a rapid linear increase, a slow increase, a plateau, and

then a decline that may be rapid. Collect at slower increase or plateau. At least 25% incorporation into DNA should be achieved.

9. Stop reaction. Add 25 µl of:

   0.02 M Na_3 EDTA
   2 mg/ml of carrier DNA (sonicated calf thymus)
   0.2% SDS

II. Deoxynucleoside Triphosphates (dNTP)

1. Cold dNTP: Final concentration in 25 µl = 20 µM.

2. [^{32}P]dNTP (usually dCTP): Wash a 1.5- or 0.5-ml polypropylene microfuge tube with ethanol, and dry the tube. Add ^{32}P-labeled dNTP in 50% ethanol to the microfuge tube. Cover tube with parafilm and punch three to four holes in film. Dry under vacuum at room temperature. Final concentration in 25 µl = 1–5 µM (~50 µCi).

III. DNase

1. Stock solution: DNase at 1 mg/ml in 50 mM Tris (pH 7.5), 10 mM $MgSO_4$, 1 mM DTT, and 50% glycerol at -20°C.

2. 0.5 µl of DNase stock solution diluted to 100 µl in dilution buffer (50 mM Tris [pH 7.5], 10 mM $MgSO_4$, 1 mM DTT, and 50 µg/ml of BSA). 0.5 µl of the diluted stock is diluted further. Dilutions are conducted at 0°C.

   a. 0.5 µl diluted to 100 µl (total dilution 1/40,000) for hybridization probe. Duplex length of probe is less than 5 kb.

   b. 0.5 µl diluted to 1 ml (total dilution 1/400,000) for recovery of labeled duplex DNA of 20 kb.

IV. Test for ^{32}P Incorporation into DNA

1. Add <0.1 µl of reaction mix into 100 µl of 500 µg/ml of carrier DNA in a disposable glass tube (5 ml).

2. Remove 10 µl and spot onto a 24-mm GFC filter.

3. a. Add 1 ml of 1 M HCl and 0.1 M Na pyrophosphate to remaining sample.

   b. Wait 10 minutes with the sample on ice.

   c. Filter onto a 24-mm GFC filter. Wash with HCl and then ethanol.

4. Dry both filters.

5. Count with hand monitor or more accurate counter.

6. Equal counts on both filters indicate 10% incorporation. Usually incorporation is 25–50%.

V. Recovery of Labeled DNA

A. 1. Load reaction on 0.7 × 20-cm Sephadex G-50 (medium) column (Bio-Rad Econo-column), preequilibrated with TE (10 mM Tris and 1 mM Na_3 EDTA at pH 7.5). Swelling time for dry G-50 is 3 hours.

2. Wash with TE (10 mM Tris and 1 mM Na_3 EDTA at pH 7.5).

3. Collect 0.5 ml of effluent samples in polypropylene tubes. DNA is usually eluted after 2 ml of wash. The location of the ^{32}P-labeled DNA in the column can be followed with a hand monitor.

4. Take first peak, ignoring tail.

B. 1. Puncture top and bottom of a 0.5-ml polypropylene centrifuge tube with a 27-gauge needle (one hole in the bottom and four holes in the top).

2. Place punctured tube in 1.5-ml polypropylene centrifuge tube with cap removed.

3. Add to inner tube: 100 µl of 50–100 mesh Bio-Gel P60 suspended in 10 mM Tris (pH 7.5), 1 mM Na_3 EDTA, and 0.2% SDS.
   Layer on top: 300–400 µl of 100–200 mesh Bio-Gel P60 suspended in the above buffer.

4. Place tube combination in clinical centrifuge (in another tube if necessary).

   a. Centrifuge 2 minutes at 1000 rpm.

   b. Add 100 µl of wash buffer: 10 mM Tris (pH 7.5), 1 mM Na_3 EDTA, and 0.2% SDS.

   c. Centrifuge 2 minutes at 1000 rpm.

   d. Transfer inner tube to clean outer tube.

   e. Dilute sample to 50 µl and load.

   f. Centrifuge 2 minutes at 1000 rpm.

   g. Add 50 µl of wash buffer and centrifuge.

   h. About 100 µl of ^{32}P-labeled DNA is collected in bottom tube.

5. If the tube is centrifuged at high speed, the P60 beads will go through the bottom hole. Check centrifuge with radioactivity hand monitor for contamination.

VI. General Remarks

The volume of the above reaction is 25 µl. Reactions of less than 10 µl have been used successfully.

Most commercial preparations of E. coli DNA polymerase I contain substantial amounts of DNase, and addition of DNase I (section III) may not be necessary.

Reference

Rigby, P.W.J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237.

What a surprise! The 1951 Phage Course participant, Waclaw Szybalski, was certainly startled when one colony of a highly mucoid, chloramphenicol-resistant E. coli mutant spread from the agar surface up to the petri-dish lid (Microbial Genetics Bull. No. 5 [1951] 10). This was aided by the newly developed gradient-plate technique (Science 116 [1952] 46–48).

PROCEDURE 23

HYBRIDIZATION TO DNA OR RNA ON SOLID SUPPORT

1. Use about 10^6 to 5×10^6 dpm of probe (nick-translated ^{32}P or ^{125}I) dissolved in TE (10 mM Tris [pH 7.5] and 1 mM Na_3 EDTA) in a polypropylene tube.

2. a. Heat at 95°C for 10 minutes to denature DNA.

   or

   b. Add 1/10 volume of 1 M NaOH, wait 10 minutes, add 1/10 volume of 1.8 M Tris-HCl and 0.2 M Tris base.

3. Place DNA- or RNA-bound filters in a heat-sealable plastic bag.

4. a. Add approximately 4 ml/100-cm^2 filter of 5× SSPE + 0.3% SDS, 100 µg/ml of denatured, sonicated carrier DNA (salmon sperm), and 50% v/v formamide (MCB).

   or

   b. Pretreat filter by placing in a heat-sealable bag and adding 4 ml/100-cm^2 filter of 50% v/v formamide (MCB), 5× SSPE, 5× BFP (1× BFP = 0.02% w/v of bovine serum albumin, Ficoll [m.w. 400,000] and polyvinyl pyrrolidone), 1% glycine, and 100 µg/ml of denatured, sonicated carrier DNA (salmon sperm). Incubate for at least 1 hour at 42°C. Remove prehybridization solution. Prepare 4 ml/100-cm^2 filter of a solution containing 50% v/v formamide, 5× SSPE, 1× BFP, 100 µg/ml of denatured, sonicated carrier DNA (salmon sperm), 10% w/v sodium dextran sulfate 500 (a 50% w/v stock solution can be prepared), and 0.3% SDS. Add half of solution to bag with the filter and mix. To remaining half, add the denatured probe, mix well, and add to bag. The sodium dextran sulfate may be omitted.

   or

   c. Add ~4 ml/100-cm^2 filter of 5× SSPE + 0.3% SDS and 100 µg/ml of denatured, sonicated carrier DNA (salmon sperm).

5. Add ~10^6 dpm denatured DNA probe. Be careful not to get any ^{32}P on the sealing area. Heat-seal the bag.

6. Place sealed bag(s) into a second bag and heat-seal. A wet paper towel placed in the second bag will help prevent drying of the filters.

7. a–b. Hybridize for 3–48 hours at 42°C for 50% formamide reaction.

   or

   c. Hybridize for 3–24 hours at 65°C for aqueous reaction.

8. Remove radioactive hybridization mix from bag. The probe may be used for additional hybridizations (see Discussion).

9. Cut open bag and remove filter.

10. a. Wash three to four times for 5–15 minutes in 250 ml of 2× SSPE + 0.2% SDS at 45°C with agitation.

    or

    b. Wash three to four times for 5–15 minutes in 250 ml of 20 mM $Na_{1.5}H_{1.5}PO_4$ + 0.2% SDS and 1 mM Na_3 EDTA at 37°C.

    or

    c. Wash three to four times for 5–15 minutes in 250 ml of 10 mM $Na_{1.5}H_{1.5}PO_4$ + 0.2% SDS and 1 mM Na_3 EDTA at room temperature.

11. Dry, cover in plastic wrap, and expose to X-ray film. The filter can be identified and oriented by including within the plastic wrap a piece of paper marked with radioactive ink. (See Procedure 24.)

100× BFP = 2% w/v bovine serum albumin, Ficoll, and polyvinyl pyrrolidone.

SSPE = 0.18 M NaCl
10 mM $(Na_{1.5})PO_4$
1 mM Na_2 EDTA
pH 7.0

1 liter of 20× SSPE

Na_2 EDTA	7.4 g	20 mM
NaOH (50%)	8.8 ml	0.16 M
$NaH_2PO_4 \cdot H_2O$	27.6 g	0.2 M
NaCl	210 g	3.6 M

Discussion

These hybridization conditions were developed for hybridizing to a unique bacterial DNA sequence with about 1 μg of total bacterial DNA eluted onto the filter from a gel. Stronger signals or shorter hybridization times can be obtained by using more labeled probe. Lower backgrounds have been obtained with ^{125}I-labeled probes by hybridizing no more than 3 hours. The reason for high backgrounds when using ^{125}I-labeled $tRNA^{Tyr}$ and long hybridizations is unknown.

If the probe is homologous to a higher-animal DNA, then something other than a salmon-sperm DNA should be used as a carrier, possibly bacterial DNA. The carrier DNA can be added to the probe, and additional carrier need not be added to the hybridization.

Either of the denaturation procedures for duplex DNA can be used. However, if the salt concentration is greater than TE, use of the alkaline denaturation is suggested. The probe can be denatured in aqueous solution with NaOH if it is to be reused following a long-term hybridization. Add 1/10 volume probe of 1.5 M NaOH, wait 10 minutes, then add 1/10 volume of 1.5 M HCl. Heat denaturation can be used for probe in formamide. The probe becomes half renatured according to the equation:

Time (hr) for half renaturation in formamide =

0.05 (vol in ml) (complexity of probe [kb])/(wt of probe [μg])

The time for optimum or sufficient hybridization is difficult to predict since one of the nucleic acids is fixed to a solid support. This is best determined empirically.

The major variable with this procedure is the nature and quality of the filters. Millipore (HAWP 000 10) or S&S BA85 nitrocellulose filters are usually used for adsorbing single-stranded DNA. However, there is variability with different lots with respect to the amount of single-stranded DNA adsorbed and the level of background hybridization.

SDS and carrier DNA are sufficient to block adsorption of labeled single-stranded DNA probe, and the BFP generally used is not necessary when conducting normal hybridizations to nitrocellulose.

PROCEDURE 24

AUTORADIOGRAPHY OF ^{32}P ON SOLID SUPPORT

1. Wrap filter in plastic wrap to avoid contamination of screens and holders.

2. Place wrapped filter in bottom of 8 × 10-inch X-ray film holder (200024 Picker cassettes). Be sure no plastic wrap protrudes from holder.

3. An intensifying screen is attached to the lid of the film holder (Dupont Cronex Lightning-Plus ZC; 224-156 without blockers, 8 × 10 in.).

4. In a dark room, place one sheet of 8 × 10-inch Kodak X-Omat R film or Dupont Cronex 4 film in the film holder. The Cronex 4 film is about one-quarter to one-half as fast as the X-Omat R film, but it is of higher resolution.

5. Close and lock film holder before turning on lights. Wrap holder in aluminum foil and place in -70°C freezer.

6. Expose for several hours to several days.

7. To develop film, remove holder from freezer and bring to room temperature before removing aluminum foil. This is to prevent condensation on film and damage to intensifying screen.

8. In a dark room, remove aluminum foil, open holder, and remove film.

9. Develop in X-ray film processor, or

    5-minute Kodak liquid X-ray developer
    1-minute stop bath (3% acetic acid)
    10-minute Kodak rapid fixer
    15-minute running water
    hang to dry

PROCEDURE 25

RECOVERY OF DNA FROM AGAROSE GELS

I. Glass Fiber Filter

1. Separate desired fragment by agarose gel electrophoresis using Tris-acetate or Tris-phosphate buffer (see Procedure 20) and 0.5 μg/ml of ethidium bromide.

2. Irradiate gel with long-wavelength UV light on only a small strip of the gel to prevent UV damage, and cut out gel slice containing DNA.

3. Add enough PPE (6 M $NaClO_4$, 50 mM $Na_{1.5}H_{1.5}PO_4$ [pH 7], and 10 mM Na_3 EDTA) to gel slice so that the final concentration of $NaClO_4$ is ≧5 M.

4. Place sample at 37°C. The gel slices will float, so it is necessary to invert sample tube periodically. Since it is essential for efficient recovery that the gel be dissolved completely, incubate for 30–60 minutes.

5. An 8-mm diameter Whatman GFC filter is placed on a scintered glass filter apparatus. A slight vacuum is used and 1 ml of PPE is applied to the GFC filter via a micropipetting device. Adjust the flow rate to approximately 0.5 ml/minute.

6. Load dissolved sample onto GFC filter; keep flow rate at about 0.5 ml/minute.

7. Rinse the sample tubes with 1 ml of PPE and apply this to the GFC filter.

8. Rinse the filter with 1 ml of PPE and then with 2–3 ml of 100% ethanol at room temperature (flow rate can be increased here if desired).

9. Remove excess liquid, but do not dry filter.

10. Fold filter in quarters and insert into a 0.5-ml microfuge tube in which a hole has been punched in the bottom with a 27-gauge needle. Tamp the filter down rather firmly to the bottom of the microfuge tube. Place the 0.5-ml tube into a 1.5-ml microfuge tube with the cap removed.

11. Elute the DNA from the filter by adding 10–20 µl of TE (10 mM Tris and 1 mM Na_2 EDTA at pH 7.5) to the filter and soak for about 1 minute. Place tubes in microfuge and spin 15 seconds. DNA is now at the bottom of the 1.5-ml tube. Repeat with 10–20 µl of TE twice. The DNA as eluted in TE is a substrate for most restriction endonucleases.

PPE = 6 M $NaClO_4$, 50 mM $Na_{1.5}H_{1.5}PO_4$ (pH 7), and 10 mM Na_3 EDTA

Discussion

1. This procedure is based on the observation that DNA sticks to GFC filters in high $NaClO_4$ concentrations.

2. The lot number of $NaClO_4$ may be important. One lot number prevented DNA binding to the filter.

3. The PO_4 in $NaClO_4$ seems to increase yields of DNA. Without PO_4, some DNA is not eluted from the GFC.

4. The capacity of an 8-mm-diameter GFC filter is 2 μg of DNA. This value appears to scale directly with the area of the filter used.

5. Yields are better for smaller DNAs. Some typical recoveries are:

DNA size (bp)	Percentage recovered
40,000	40–65
10,000	50–75
5,000	50–80
≦ 1,000	50–90

References

Chen, C.W. and C.A. Thomas, Jr. 1980. Recovery of DNA segments from agarose gel. Anal. Biochem. 101:339.

McDonell, M. and T. St. John (pers. comm.).

II. KI Equilibrium Density Gradient

1. Separate desired fragment by agarose gel electrophoresis using Tris-acetate or Tris-phosphate buffer (see Procedure 20) and 0.5 μg/ml of ethidium bromide.

2. Irradiate gel with long-wavelength UV light on only a small strip of the gel to prevent UV damage, and cut out and weigh the gel slice containing DNA.

3. Dissolve gel in a saturated solution of KI.

4. Add ethidium bromide to give a final concentration of about 50 μg/ml.

5. Adjust density to 1.5 g/ml.

   Refractive index $\eta_{20} = 0.1731 \cdot \rho + 1.1617$ (valid for $1.3 \leqq \rho \leqq 1.7$)

6. Centrifuge to equilibrium at 30,000 rpm for 24–48 hours at 20°C.

Reference

Blin, N., A.V. Gabain, and H. Bujard. 1975. Isolation of large molecular weight DNA from agarose gels for further digestion by restriction enzymes. FEBS Lett. 53:84.

III. Electroelution of DNA into Hydroxylapatite

1. Separate desired fragment by agarose gel electrophoresis using Tris-acetate buffer (Procedure 20) containing 0.5 µg/ml of ethidium bromide. Remove enough buffer from apparatus so that gel surface is above buffer.

2. Visualize bands by using long-wavelength UV light and cut out a 1–2-mm well just ahead of the band.

3. Fill the well with a thick slurry (in Tris-acetate running buffer) of Bio-Rad HTP hydroxylapatite. Fill to capacity, adding more slurry as solid settles.

4. Gently add buffer to cover gel and continue electrophoresis, using long-wavelength UV light to monitor the movement of the band from the gel into the hydroxylapatite.

5. When the band disappears from the gel, stop electrophoresis. Using a pasteur pipette, remove the slurry into another pasteur pipette plugged with siliconized glass wool and set up on a ringstand to form a mini-column.

6. Wash the column with 5 volumes (usually this means 5 × 200 µl) of 10 mM KPO_4 buffer (pH 6.8) and 0.5 µg/ml of ethidium bromide. Wash again with 5 volumes of 100 mM KPO_4 buffer and 0.5 µg/ml of ethidium bromide.

7. Elute DNA with 400 mM KPO_4 buffer containing 0.5 µg/ml of ethidium bromide. Follow elution by taking small (~50 µl) fractions and observing fluorescence with long-wavelength UV light.

8. Pool fractions containing DNA and extract with buffer-saturated phenol.

9. Remove aqueous phase and dialyze overnight against 10 mM Tris-HCl (pH 8) and 1 mM Na_3 EDTA.

Reference

Tabak, H.F. and R.A. Flavell. 1978. A method for the recovery of DNA from agarose gels. Nucleic Acids Res. 5:2321.

II. Plastic Wrap and Ring Method

1. Stretch plastic wrap over a ring (e.g., the rim of a plastic petri dish with the bottom removed).

2. Spot samples of DNA in equal volumes of 1 μg/ml of ethidium bromide on the plastic wrap and compare as above in method I.

3. Method II works best for pure DNA, and method I is best for DNA with interfering components of low molecular weight.

PROCEDURE 27

ELECTRON MICROSCOPY OF DNA

I. Aqueous Procedure

A. Spreading method

1. Spreading solution. Use 30 µl:

   5 µl of 5 M NH_4Ac and 1 mg/ml of cytochrome c
   25 ng of DNA
   H_2O to make final volume of 50 µl

2. Hypophase (0.25 M NH_4Ac).

B. Drop method

1. 3.5 µl of 5 M NH_4Ac and 1 mg/ml of cytochrome c

2. 25 ng of DNA

3. H_2O to make a final volume of 50 µl. Touch grid to side of drop on Teflon or other nonwetting surface. Best for small DNA (ϕX174 and SV40).

C. Stain

1. Add 10 µl of UO_2Ac_2 (0.05 M UO_2Ac_2 and 0.05 M HCl in H_2O) to 10 ml of 90% ethanol.

2. Keep covered and out of light.

3. Stain grids for 30 seconds.

4. Rinse 5 seconds in 90% ethanol.

References

Davis, R.W., M.N. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. 21D:413.

Ferguson, J. and R.W. Davis. 1978. Quantitative electron microscopy of nucleic acids. In Advanced techniques in biological electron microscopy II (ed. J.K. Koehler), p. 123.

II. Formamide Procedure

A. 1. Spreading solution. Use 50 µl of:

10 µl of 1 M Tris-HCl (pH 8.5), 0.1 M Na_2 EDTA, and 0.5 mg/ml of cytochrome c
25 ng of DNA
H_2O to give volume of 60 µl
40 µl of formamide

2. Hypophase (made within 5 minutes of use)

0.01 M Tris and 10^{-3} M Na_2 EDTA (pH 8.5) (100 ml)
10% formamide (10 ml)

B. Stain

1. Add 10 µl of UO_2Ac_2 stock (0.05 M UO_2Ac_2 and 0.05 M HCl in H_2O) to 10 ml of 90% ethanol.
2. Keep covered and out of light.
3. Stain grids for 30 seconds.
4. Rinse 5 seconds in 90% ethanol.

C. Isodenaturing Conditions (for sets of spreading and hypophase solutions)

	Spreading	Hypophase
1.	30% formamide 0.1 M Tris (pH 8.5) 0.01 M Na_2 EDTA	5% formamide 0.01 M Tris (pH 8.5) 0.001 M Na_2 EDTA
2.	40% formamide 0.1 M Tris (pH 8.5) 0.01 M Na_2 EDTA	10% formamide 0.01 M Tris (pH 8.5) 0.001 M Na_2 EDTA
3.	50% formamide 0.1 M Tris (pH 8.5) 0.01 M Na_2 EDTA	20% formamide 0.01 M Tris (pH 8.5) 0.001 M Na_2 EDTA
4.	60% formamide 0.1 M Tris (pH 8.5) 0.01 M Na_2 EDTA	30% formamide 0.01 M Tris (pH 8.5) 0.001 M Na_2 EDTA

5.	70% formamide 0.1 M Tris (pH 8.5) 0.01 M Na_2 EDTA	40% formamide 0.01 M Tris (pH 8.5) 0.001 M Na_2 EDTA
6.	80% formamide 0.05 M Tris (pH 8.5) 0.005 M Na_2 EDTA	50% formamide in distilled water

References

Davis, R.W. and R.W. Hyman. 1971. A study in evolution: Homology between coliphages T7 and T3. J. Mol. Biol. 62:287.

Davis, R.W., M.N. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. 21D:413.

PROCEDURE 28

GENERAL PROCEDURE FOR HETERODUPLEX FORMATION

I. Heteroduplex

1. 20 µl of H_2O (minus the volume of phage or DNA).
2. 0.1 µg of each DNA (as phage).
3. 0.5 µl of 1 M Na_4 EDTA.
4. 2.5 µl of 1 M NaOH.
5. Wait 10–30 minutes at 25°C.
6. 2.5 µl of 1.8 M Tris-HCl and 0.2 M Tris base (pH 7.2).
7. 25 µl of formamide.
8. Incubate at 25–40°C for 20–60 minutes.

II. Electron Microscopy of Heteroduplex

A. Spreading Solution

1. 10 µl of heteroduplex preparation.
2. 10 µl of 1 M Tris, 0.1 M Na_2 EDTA (pH 8.5), and 0.5 mg/ml of cytochrome c.
3. 45 µl of H_2O.
4. 35 µl of formamide.

5. Spread 50 µl.

B. Hypophase (made within 5 min of use)

0.01 M Tris and 10^{-3} M Na_2 EDTA (pH 8.5)
10% formamide

References

Davis, R.W. and N. Davidson. 1968. Electron-microscopic visualization of deletion mutations. Proc. Natl. Acad. Sci. 60:243.

Davis, R.W., M.N. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. 21D:413.

Ferguson, J. and R.W. Davis. 1978. Quantitative electron microscopy of nucleic acids. In Advanced techniques in biological electron microscopy II (ed. J.K. Koehler), vol. 2, p. 123. Springer-Verlag, Berlin.

Westmoreland, B.C., W. Szybalski, and H. Ris. 1969. Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science 163:1343.

PROCEDURE 29

PREPARATION OF ENZYME FRACTIONS FROM λcI857 Sam7 LYSOGENS

I. General Procedure

A. Growth and Induction

Grow cells at 30°C to A_{600} of 0.6 (or 1.0 with forced aeration) in LB broth or Ardamine Z yeast extract-Cerelose B1 (Panasenko et al. 1978). Heat to 42°C for 15–20 minutes, and grow cells for 2 1/2–3 hours at 37°C. Maintain pH >7.0 during growth.

B. Harvest

Centrifuge cells, weigh cell paste, and resuspend at 100-fold concentration in 50 mM Tris-HCl (pH 7.5) + 10% sucrose. Drip into liquid nitrogen to freeze, and store at -70°C.

C. Lysis

Follow procedure for heat lysis (Scott and Kornberg 1978). Thaw cells and add Tris-sucrose for a final concentration of 150 g cells/liter of lysis buffer. Leave enough room to add:

ammonium sulfate to 5% saturation
spermidine-Cl_3 to 20 mM
Na_2 EDTA to 20 mM
DTT to 1 mM

Bring pH to 8 with solid or 2 M Tris base. Add lysozyme to 0.2 mg/ml and incubate on ice for 30 minutes. Heat in 250-ml batches in a 37°C water bath for about 5 minutes (sample will not reach 37°C, but only about 15°C) to complete lysis.

Centrifuge lysate for 3 hours at 20,000 rpm in a JA-20 rotor, 90 minutes at 25,000 rpm in an SW 27 rotor, or 60 minutes at 40,000 rpm in a 60 Ti or 45 Ti rotor.

References

Panasenko, S.M., R.J. Alazard, and I.R. Lehman. 1978. A simple three-step procedure for the large scale purification of DNA ligase from a hybrid λ lysogen constructed in vitro. J. Biol. Chem. 253:4590.

Scott, J.F. and A. Kornberg. 1978. Purification of the rep protein of Escherichia coli. J. Biol. Chem. 253:3292.

II. Purification of DNA Polymerase I from E. coli 594 Lysate
(see Strain List)

A. Fractionation

Add solid ammonium sulfate (0.25 g/ml) to the 5% ammonium sulfate supernatant. Stir for 1 hour at 4°C and centrifuge at 10,000 rpm for 30 minutes in a JA-14 rotor. Discard pellet. Add solid ammonium sulfate (0.16 g/ml) to the 47% supernatant, stir, and centrifuge (Richardson et al. 1964). Resuspend pellet in 200 mM $NaPO_4$ (pH 6.5) and 1 mM DTT. Adjust ionic strength and pass through DE-52 column (0.5–1.0 ml/g of cells) in that buffer.

B. Phosphocellulose Chromatography

Dialyze DE-52 flowthrough into 20 mM KPO_4 (pH 6.5) and 1 mM DTT. Load a P-11 column (0.5–1.0 ml/g of cells) equilibrated in the same buffer. Wash with 1 column volume of the 20 mM buffer and 1 volume of 50 mM buffer (1 mM DTT), and elute with a 7–8-column-volume gradient of 50–250 mM buffer (1 mM DTT). Pool peak fractions (central protein peak in gradient) and precipitate with ammonium sulfate (85% saturation [0.6 g/ml]). Resuspend in 100 mM KPO_4 (pH 7.0) at 10 mg/ml of protein.

C. G-100 Sephadex Chromatography

Equilibrate a G-100 column (bed volume $\geq$ 25× sample volume) in column buffer (100 mM KPO_4 [pH 7.0]). Load and follow with column buffer. Pool peak fractions, which will be just behind the void volume. Concentrate as above, if desired. Store frozen in the final column buffer at -70°C (Jovin et al. 1969); or for storage of small sample for about 1 year, add equal volume of glycerol and store at -20°C.

D. Assay

50 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT, 100 µg/ml of activated (sonicated) calf-thymus DNA or salmon-sperm DNA, 20 µM each dNTP, and about 100,000 cpm [α-^{32}P]dCTP or dGTP. One unit is about 10% incorporation into acid-precipitable material in 3 minutes at 37°C in a 100-µl reaction. (10-nmole incorporation in 30 min, corrected for base composition.) Pure enzyme is about 2500 units/mg in this assay.

References

Jovin, T.M., P.T. Englund, and L.L. Bertsch. 1969. Enzymatic synthesis of deoxyribonucleic acid. J. Biol. Chem. 244:2996.

Richardson, C.C., C.L. Schildkraut, H. Vasken Aposhian, and A. Kornberg. 1964. Enzymatic synthesis of deoxyribonucleic acid. J. Biol. Chem. 39:222.

III. T4 DNA Ligase from E. coli E1150 Lysate (see Strain List)

A. Fractionation

Add solid ammonium sulfate (0.33 g/ml) to the 5% supernatant, stir for 1 hour in the cold, and centrifuge at 10,000 rpm for 30 minutes in a JA 14 rotor. Resuspend the pellet in 25 mM Tris (pH 7.2), 0.3 M NaCl, and 1 mM DTT. Adjust ionic strength to 0.3 M NaCl and pass through DE-52 column (0.5–1.0 ml/g of cells) in that buffer.

B. P-11 and DE-52 Fractionation

Dialyze sample into buffer (25 mM Tris [pH 7.2] and 1 mM DTT) and load P-11 column (0.5–1.0 ml/g of cells) equilibrated with same buffer. Wash with 2 column volumes of buffer and 2 volumes of buffer + 0.15 M NaCl, and elute with 0.75 M NaCl buffer. Dialyze eluted protein into 25 mM Tris (pH 7.2) and 1 mM DTT. Centrifuge any precipitated protein and discard. Load onto a DE-52 column (0.5 ml/mg of protein) and wash with 2 volumes of 25 mM (pH 7.2) and 1 mM DTT. Elute with this buffer + 0.3 M NaCl.

C. HAP Chromatography

Load DE-52 elution directly onto a HAP column (0.5 ml/mg of protein) equilibrated with 25 mM Tris (pH 7.2), 1 mM DTT, and 0.3 M NaCl. Wash with 2 column volumes of 0.3 M NaCl buffer and elute with a 0–0.5 M ammonium sulfate gradient in 0.3 M NaCl buffer. The single major protein peak contains the ligase activity (>95% pure).

D. Assay

An assay that is less than quantitative but convenient is cyclization of a small circular DNA molecule cleaved with a phosphatase-

and exonuclease-free preparation of the restriction endonuclease that generates cohesive ends. For more traditional assays, see Weiss et al. (1968) or Modrich and Lehman (1970). Most assays probably will not work properly until the first DE-52 step is completed, but will certainly work after completion of the P-11 step.

References

Modrich, P. and I.R. Lehman. 1970. Enzymatic joining of polynucleotides. J. Biol. Chem. 245:3626.

Weiss, B., A. Jacquemin-Sablan, T.R. Live, G.C. Fareed, and C.C. Richardson. 1968. Enzymatic breakage and joining of deoxyribonucleic acid. J. Biol. Chem. 243:4543.

SECTION III

APPENDIXES

APPENDIX 1

MEDIA, DRUG CONCENTRATIONS, AND NUTRITIONAL SUPPLEMENTS

I. Media

A. LB (Luria-Bertani) Medium

Per liter:	Bacto-tryptone	10 g
	Bacto-yeast extract	5 g
	NaCl	5 g
For plates:	Bacto-agar	15 g
For top agar:		7 g

B. λ Medium

Per liter:	Bacto-tryptone	10 g
	NaCl	5 g
For plates:	Bacto-agar	12 g
For top agar:		7 g

C. Agarose Media (for quick DNA preparations)

Instead of Bacto-agar in the standard recipes, use 10 g/liter of agarose for bottom agar and 6 g/liter of agarose for top agar.

D. Green Indicator Plates

Per liter:	Bacto-tryptone	8 g
	Bacto-yeast extract	1 g
	NaCl	15 g
	Bacto-agar	15 g

Autoclave, and then add sterile:

40% glucose	34 ml
2.5% alizarin yellow	25 ml
2% aniline blue	6.6 ml

Alizarin yellow G, GG is the product of MCB, and aniline blue is from Fisher. The alizarin yellow solution must be heated just before use as the dye is insoluble at room temperature.

E. Red Plates (for scoring production of β-lactamase)

Per liter:	trypticase (BBL)	10 g
	NaCl	5 g
	Bacto-agar	10 g

Autoclave, and then add:

1–5 mg of ampicillin, depending on the lot. The intent is to just barely inhibit sensitive cells.
25 mg of 2,3,5 triphenyl tetrazolium chloride
33 ml of sterile 30% galactose

F. Superbroth

Per liter:	Bacto-tryptone	33 g
	Bacto-yeast extract	20 g
	NaCl	7.5 g
	10 M NaOH	3.5 ml

G. Trypticase-EDTA

Per liter:	trypticase (BBL)	10 g
	NaCl	5 g
For plates:	Bacto-agar	10 g
For top agar:		6.5 g

H. E Medium (Vogel-Bonner minimal)

To prepare 1 liter or 50× E, dissolve each of the following salts (in the order given) in water at a temperature of at least 45°C. Store over chloroform and sterilize when diluted.

Per liter 50× E:	distilled water	670 ml
	$MgSO_4 \cdot 7H_2O$	10 g
	citric acid$\cdot 1H_2O$	100 g
	K_2HPO_4 anhydrous	500 g
	$NaHNH_4PO_4 \cdot 4H_2O$	175 g

For medium: Dilute 50-fold, sterilize, and add sugars and supplements as required.

For plates: Make up equal volumes of 2× E and 3% Bacto-agar, autoclave separately, and add sugars and supplements as required.

I. NCE Medium (No carbon or no citrate E medium)

This medium is used when a carbon source other than glucose or citrate is to be used in a selective medium. Since Salmonella can utilize the citrate present as a chelating agent in E medium, a citrate-free minimal medium must be used. The NCE medium can be made up as a 50-fold concentrate if $MgSO_4$ is left out of the concentrate and is added separately to the final medium.

50× NCE (1 liter):	KH_2PO_4	197 g
	$K_2HPO_4 \cdot H_2O$	325.1 g
	$Na(NH_4)HPO_4 \cdot H_2O$	175 g
	H_2O	925 ml
1000× (1 M $MgSO_4$ [for NCE medium])	$MgSO_4$	265.5 g
	H_2O	1000 ml

To make 2× NCE medium, mix:

1 liter H_2O
40 ml 50× NCE concentrate
2 ml 1000× $MgSO_4$

Autoclave the above mixture, add to an equal volume of molten 2× (3%) agar, add a carbon source (usually at a final concentration of 1%), and pour the plates.

J. M9 Medium

Per liter:	$Na_2HPO_4 \cdot H_2O$	6 g
	KH_2PO_4	3 g
	NaCl	0.5 g
	NH_4Cl	1 g

Autoclave, and then add sterile:

1 M $MgSO_4$	1 ml
0.01 M $CaCl_2$	10 ml

($CaCl_2$ can be omitted when using ordinary unpurified agar.)

For plates: Make up equal volumes of 2× M9 and 3% Bacto-agar, autoclave separately, mix, and add sugars (e.g., 0.2% glucose or maltose) and supplements as required.

For M9 top agar, mix equal volumes of 2× M9 and 1.3% Bacto-agar, autoclaved separately. Sometimes, some fine precipitate will form after long storage (or reheating) of M9 top agar. This precipitate is harmless.

K. MacConkey Plates

Per liter: MacConkey agar base (Difco) 40 g

Autoclave, and then add sugar to 1%. For lactose, Difco sells medium already containing the sugar.

L. EMB Plates

Per liter:

EMB agar base (Difco)	27.5 g
or	
Bacto-tryptone	10 g
Bacto-yeast extract	1 g
NaCl	5 g
KH_2PO_4	2 g
Bacto-agar	15 g

Autoclave, and then add sterile:

4% eosin yellow	10 ml
0.65% methylene blue	
sugar to make 1%	10 ml

M. Bochner Selection Plates (for selection of Tet^S mutants)

Solution A:	Bacto-tryptone	10 g
	Bacto-yeast extract	5 g
	Chlortetracycline·HCl	50 mg
	agar	15 g
	H_2O	500 ml
Solution B:	NaCl	10 g
	$NaH_2PO_4 \cdot H_2O$	10 g
	glucose	2 g
	H_2O	500 ml

Autoclave solutions A and B separately for 20 minutes at 15 psi (timing is important). Mix and cool to pouring temperature. Add 5 ml of $ZnCl_2$ (20 mM) and either 6 ml of fusaric acid (2 mg/ml) or 10 ml of quinaldic acid (10 mg/ml).

N. Stab Agar

Per liter:	nutrient broth (Difco)	8 g
	Bacto-agar	6 g

Autoclave (or heat in microwave) and mix thoroughly. Dispense into vials and then autoclave to sterilize. It is not a good idea to try to dispense sterile medium into sterile vials without resterilizing.

O. λ YM

This medium consists of λ broth + 0.01% yeast extract and 0.2% maltose. Although it appears frequently in the literature, it can usually be replaced with TYM.

P. TYM Broth

LB broth made 0.2% in maltose by adding 1/100 volume of sterile 20% maltose.

Q. λ dil

10 mM Tris-HCl (pH 7.5) and 10 mM $MgSO_4$. For long-term storage of λ stocks, the addition of 50 mM NaCl and .01% gelatin is sometimes recommended, especially when the phage has been purified in CsCl.

R. Buffered Saline

0.85% (w/v) NaCl and 0.066 M $NaPO_4$ buffer (pH 7.0).

II. Drug Concentrations

In general, rich media contain more drug than minimal, with the exception of plates to be used in DNA transformation. The recommended final concentrations are: 20 μg/ml of tetracycline and 50 μg/ml of ampicillin in rich media and 10 μg/ml of tetracycline and 25 μg/ml of ampicillin in minimal media and media for transformation experiments.

III. Nutritional Supplements

Below is a list of nutritional supplements frequently used in bacterial work. Stock solutions are such that 5 ml of the solution added to 1 liter of medium will result in the appropriate concentration. Limiting concentration is the concentration at which auxotrophs requiring that supplement will form tiny colonies distinguishable from wild type. All stock solutions are such that 5 ml is added per liter of medium for normal supplementation level.

Nutrient	Plate conc. (mM)	Low conc. (mM)	Stock sol. (%)	ml/liter for low (%)	Sterilize	Remarks
Adenine	5.0	0.001	1.35		F	0.1 N HCl
Adenosine	5.0	0.001	2.67		A	
Alanine	0.47		0.84		A	
Arginine	0.6	0.01	2.53	0.86	A	
Asparagine	0.32		0.84		F	
Aspartate·K	0.3		1.0		F	
Biotin	0.1		0.49		A	
Cysteine	0.3		0.73		F	
Diaminopimelic acid (DAP)	0.1		0.38		A	
Glutamate·Na	5.0				F	
Glutamine	5.0		14.6		A	
Glycine	0.13		0.2		A	
Guanine	0.3		0.91		A	in 1 N HCl
Guanosine	0.3		1.7		A	
Histidine	0.1	0.005	0.31	0.25	A	
Histidinol	1.0		4.28		A	
Isoleucine	0.3		0.79		A	
Leucine	0.3	0.005	0.79	0.086	A	
Lysine	0.3	0.005	1.1	0.086	A	
Methionine	0.3		0.9	0.086	A	
Nicotinic acid	0.1		0.25		A	
Pantothenate·Ca	0.1		0.48		A	
Phenylalanine	0.3		0.99		A	0.01 N HCl
Proline	2.0	0.002	4.6	0.005	A	
Pyridoxine·HCl	0.1		0.41		A	
Serine	4.0	0.01	8.4	0.0125	A	
Thiamine	0.05		0.337		A	
Threonine	0.3		0.71		A	
Thymine	0.32		0.81		A	
Tryptophan	0.1		0.41		F	
Tyrosine	0.1		0.36		F	
Uracil	0.1	0.003	0.224	0.15	A	
Uridine	0.1	0.003	0.488		A	
Valine	0.3		0.7		A	
EGTA	10.0		2 M		A	neutralize

APPENDIX 2

DIAGNOSIS OF AUXOTROPHS (AUXANOGRAPHY)

The composition of these plates is described in the following table. All nutrients are used at the final concentrations given in Appendix 4. The compositions of Media 1–5 are listed vertically in the table. The compositions of Media 6–10 are listed horizontally. Medium 11 is an assortment of compounds not included in the others; its contents are listed horizontally at the bottom of the table. Some notes on the use of these media follow the table.

	1	2	3	4	5
6	adenosine	guanosine	cysteine	methionine	thiamine
7	histidine	leucine	isoleucine	lysine	valine
8*	phenylalanine	tyrosine	tryptophan	threonine	proline
9	glutamine	asparagine	uracil	aspartic acid	arginine
10	thymine	serine	glutamic acid	DAP	glycine
11	pyridoxine, nicotinic acid, biotin, pantothenate, alanine				

* See note 5 in Discussion below.

Discussion

1. Some purine mutants grow on adenosine or guanosine; they will grow on pools 1, 2, and 6.

2. Some purine mutants require adenosine + thiamine; they will grow only on pool 6.

3. pyrA mutants require uracil + arginine; they grow on pool 9.

4. Mutants requiring isoleucine + valine will grow only on pool 7. When using E. coli K12, pool 5 must have isoleucine added, as all K12 strains are sensitive to valine in the absence of isoleucine.

5. Mutants with early blocks in the aromatic pathway will only grow on pool 8. In addition to the nutrients listed, pool 8 contains PABA and DHBA to satisfy mutants blocked in new synthesis of aromatic amino acids.

6. Early blocks in the lysine pathway grow only on pool 4.

7. Pool 11 is a catchall, mostly vitamins.

8. Solutions of the above nutrient pools (1–11) can be made up as a tenfold concentrate over the final concentration used in media.

9. Use salts of glutamic and aspartic acids.

10. Do not autoclave glutamine or asparagine solutions.

11. Keep solutions containing tryptophan dark.

12. Pool 9 contains 20 mM glutamine; pool 1 contains 5 mM. High-glutamine requirers (such as glnA mutants) will grow only on pool 9.

APPENDIX 3

STORAGE OF BACTERIA, PHAGE, AND DNA

I. A. Storage of Bacteria

Two storage methods are in general use. Room temperature stab vials are satisfactory for routine storage of auxotrophs (including Tn*10* insertions) and multiply-marked strains with fairly stable genotypes. This method is not satisfactory for Tn*5* insertion mutants, which accumulate additional copies of Tn*5*. For such mutants, for Hfrs, and for strains carrying unstable genomes, the low-temperature (-70°C) method (described below) is recommended.

B. Storage of Phage

P22 lysates (Procedure 2) are generally stable at 4°C in a well-buffered medium, including calcium and magnesium. Lysates of P22 (HT124, the high-frequency transducing mutant) are somewhat less stable, probably due to the effects of the HT mutation on capsid structure. λ lysates (see Procedure 2) can be stored at 4°C over one drop of $CHCl_3$. However, the titer may drop one log per year. Long-term storage of P22 (HT) and of λ stocks is at low temperature, as outlined below.

II. Storage Procedures

A. Room-temperature Stab Vials

Vials: 0.5-dram capacity, 12 × 35 mm
Caps: Bakelite with rubber liners
Labels: Avery (Kum Klean) circular labels for room temperature. Time Tapes and Shamrock Tapes sell circular labels with low-temperature adhesive for use at -70°C.

Medium:	nutrient broth (Difco)	2 g
	agar	1.5 g
	H_2O	250 ml

B. Preparation of Vials

The medium is heated until the agar is fully melted. Dispense into vials (1.2 ml/vial) with a repeating syringe. Cap vials and then autoclave (no breakage occurs). Vials are stored at 4°C until used.

C. Use of Vials

Stab the strain to be preserved into the soft agar with an applicator stick or inoculating needle. Dip threads at top of vial into melted paraffin and screw the cap on tightly over the soft wax. The waxing procedure is done to prevent drying. Vials such as these have maintained cultures for over 10 years.

D. Low-temperature Storage of Phage and Bacteria

Both phage and bacteria can be stored in 7% DMSO (or 15% glycerol) at -70°C. In practice, sterile tubes are prepared containing 70 µl of DMSO (or 150 µl of glycerol). One milliliter of phage suppression or fresh bacterial culture is pipetted into the vial and mixed. The vial is capped and placed at -70°C.

To remove phage or cells from storage, the vial need not be thawed. Open the vial and scratch the surface of the frozen material with a sterile needle. Streak onto the appropriate medium or host strain. The storage vial can be recapped and returned to -70°C.

III. Storage of DNA

The following properties of reagents and conditions are important considerations in processing and storing DNA and RNA.

1. Heavy metals promote phosphodiester breakage.

2. Na_2 EDTA is an excellent heavy metal chelator.

3. Citrate has no buffer capacity above pH 7 and is a poor metal chelator. The use of citrate is purely historical and provides no obvious benefits; therefore, we have abandoned its use.

4. Free radicals are formed from chemical breakdown and radiation. They cause phosphodiester breakage.

5. UV: 260-nm irradiation causes a variety of lesions, including thymine dimers and cross-links. Biological activity is lost rapidly. 320-nm irradiation can also cause cross-links, but less efficiently.

6. Diethyl pyrocarbonate or diethyloxydiformate carbethoxylates single-stranded nucleic acids and causes a loss of biological activity. It generally does not react with duplex nucleic acids at room temperature.

7. Low pH causes depurination but has a high activation energy.

8. Ethidium bromide causes photooxidation of DNA with visible light and molecular oxygen. It is a good free-radical scavenger.

9. Phenol: Oxidation products can cause phosphodiester breakage.

10. Ether: Phosphodiester breakage is probably caused by peroxides.

11. Formamide: Most aqueous formamide solutions will become acidic (pH 5) with time. Since the reaction is alkaline-catalyzed, it is best to buffer at pH 7 with phosphate or PIPES. RNA shows some phosphodiester breakage on contact over several days.

12. Ethanol: If no heavy metals are present, ethanol does not damage DNA.

13. Nucleases are found on human skin; therefore, avoid direct or indirect contact between nucleic acids and fingers. Most DNases are not very stable; however, many RNases are very stable. RNase can adsorb to glass or plastic and remain active.

14. 5°C: This temperature is one of the best and simplest conditions for storing DNA.

15. -20°C: This temperature causes extensive single- and double-strand breaks. The salt in the DNA freezes out, and the freezing point of a saturated salt solution is about that of the cycling temperatures of most refrigerator-freezers. Thus, the DNA is exposed to frequent freeze-thaw cycles.

16. -70°C: This temperature is probably excellent for long-term storage. Remember that free-radical damage can be much greater in the frozen state.

For long-term storage of DNA, it is best to store in high salt (≧ 1 M) in the presence of high Na_2 EDTA (≧ 10 mM) at pH 8.5 (Tris).

Storage of DNA in buoyant CsCl with ethidium bromide in the dark (wrapped in aluminum foil) at 5°C is excellent. There is about one phosphodiester break per 200 kb of DNA per year.

Storage of λ DNA in the phage is better than storing the pure DNA. The DNA can be stored in the phage in buoyant CsCl for 5 years with no detectable breakdown (< one break/200 kb).

APPENDIX 4

BUFFERS AND SOLUTIONS

I. Buffers

Buffer	pK_a (20°C)	ΔpK_a/°C	Mol. wt.	Molarity at sat. 0°C
PIPES	6.80	-0.0085	342	1.4
MOPS	7.20	-0.006	209	3.0
TES	7.50	-0.020	229	2.6
HEPES	7.55	-0.014	238	2.2
HEPPS	8.00	-0.007	252	2.5
Tris	8.30	-0.031	121	2.4

Reference

Good, N.E., G.D. Winget, W. Winter, T.N. Connolly, S. Izawa, and K.M.M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry 5:467.

Tris·HCl

pH	Tris-HCl (moles)	Tris-OH (moles)
7.2	0.889	0.111
7.3	0.867	0.133
7.4	0.837	0.163
7.5	0.804	0.196
7.6	0.767	0.233
7.7	0.724	0.276
7.8	0.673	0.327
7.9	0.618	0.382
8.0	0.562	0.438
8.1	0.509	0.491
8.2	0.448	0.552
8.3	0.389	0.611
8.4	0.334	0.666
8.5	0.280	0.720
8.6	0.232	0.768
8.7	0.190	0.810
8.8	0.156	0.844
8.9	0.122	0.878
9.0	0.096	0.904

pH of Buffer (using equal molar amounts of the acid and base form)

HCl/KCl = 1.4
Glycine HCl/glycine = 2.5
Formic acid/Na formate = 3.7
Citric acid/Na_3 citrate = 4.6
Acetic acid/Na acetate = 4.7
Cacodylic acid/Na cacodylate = 6.2
KH_2PO_4/Na_2HPO_4 = 6.9
NH_4Cl/NH_4OH = 9.4
Glycine/Na glycinate = 9.7
$NaHCO_3/Na_2CO_3$ = 10.4

pH Standards at 25°C

0.1 M HCl = 1.10
Sat. KH tartrate = 3.56
25 mM KH_2PO_4 + 25 mM Na_2HPO_4 = 6.86
0.01 M Borax = 9.18
Sat. $Ca(OH)_2$ = 12.45
0.1 M NaOH = 12.88

Protein Solutions

$A_{280} \cong 1.0$ for 1 mg/ml

T4 DNA ligase $A_{280}^{0.1\%} = 1.0$

E. coli DNA polymerase $A_{280}^{0.1\%} = 0.85$

Protein concentration in mg/ml = $1.5\ (A_{280}) - 0.75\ (A_{260})$ if contaminated with nucleic acid

APPENDIX 5

PREPARATION OF DIALYSIS TUBING

1. Cut to convenient lengths (10–20 cm).

2. Boil in $NaHCO_3$ buffer:

   1 T baking soda per quart of water
   1/2 t Na_2 EDTA per quart of water

3. Wash inside of tubing with distilled water.

4. Boil in 10^{-3} M Na_3 EDTA.

5. Store at 5°C.

6. Before use, wash inside of tubing with distilled water.

7. Always handle tubing with gloves.

APPENDIX 6

WEIGHTS AND MEASURES

I. Microliter Volume Measurement

A. Gilson Pipetman (P20 and P200)

The P20 measures from 1 µl to 20 µl, and the P200 measures from 20 µl to 200 µl. There is a large dead-air space in the pipette tip. If it is submerged in a liquid that is warmer or colder than the air space in the pipette tip and the submerging causes a temperature change in this air space, the volume of liquid removed will not be correct. Always allow the plunger to slowly return to the "up" position; never permit it to snap up. Wait 1–2 seconds to ensure that full volume is in tip.

B. Microliter Syringes

1. 100-µl Hamilton 710-SN with 20-gauge, 6-mm, flat-end needle.

2. 50-µl Hamilton 705-SN with 20-gauge, 6-mm, flat-end needle.

3. 10-µl Glenco 19909-10-2 × 26 with 26-gauge, 6-mm, flat-end needle.

4. 1-µl Hamilton 7101 N.

The 100- and 50-µl syringes use I.D. 0.034-inch polyethylene or Teflon thin-walled tubing. Intermedic PE 90 (Clay Adams) is generally used. The 10- and 1-µl syringes use I.D. 0.015-inch polyethylene or Teflon thin-walled tubing. Intermedic PE20 (Clay Adams) is generally used. The 1-µl syringe is most

often used to measure small amounts of enzyme. In general, removal of 0.1 µl has an accuracy of ±20%.

These syringes have a smaller dead air space and, in general, are more accurate. The wire plunger in the 100-, 50-, and 10-µl syringes (but not the 1-µl) should be cleaned periodically. Remove the plunger and rinse the barrel and plunger with acetone and then water. If the plunger becomes too dirty, it will stick and/or split the barrel. The splits are usually hairline fractures seen by refracted light and will cause the syringe to leak air, giving inaccurate volume measurements. If extensive wear causes air to leak around the plunger, lubricate the barrel and plunger with a little glycerol.

II. Melting Temperature of DNA

1. Base Composition and Salt

$$\underline{T}_m = 16.6 \log (Na^+) + 0.41 (\%G + C) + 81.5 \qquad \text{(eq. 1)}$$

for λb2 DNA %G + C = 51%

0.1 M NaCl $\underline{T}_m$ = 85.8°C

0.5 M NaCl $\underline{T}_m$ = 97.4°C

2. Formamide

1% formamide lowers $\underline{T}_m$ by 0.65°C.

3. DNA Sequence Homology

For every 1% lowering of homology, the $\underline{T}_m$ is lowered by 1.5°C.

4. Length of DNA

The lower melting temperature due to the shortening of duplex length is calculated by:

$$\underline{T}_m \text{ of short duplex} = \underline{T}_m \text{ of long duplex (eq. 1)} - \frac{500}{\text{no. of base pairs in short duplex}}$$

III. Clearing Time

JA-21		JA-20		JS-13		JA-14		JA-10		JS-7.5	
18 × 10 ml		8 × 50 ml		4 × 50 ml		6 × 250 ml		6 × 500 ml		4 × 250 ml	
(K rpm)	(C)	(K rpm)	(C)	(K rpm)	(C)	(K rpm)	(C)	(K rpm)	(C)	(K rpm)	(C)
2	580	2	770	2	800	2	820	2	900	2	1200
4	140	4	190	4	200	4	200	3	400	3	520
5	92	5	120	5	130	5	130	4	230	4	290
6	64	6	86	6	90	6	90	5	140	5	190
8	36	8	48	8	50	8	51	6	100	6	130
10	23	10	31	10	32	10	32	7	74	7	95
12	16	12	21	11	26	11	27	8	56	7.5	83
14	12	14	16	12	22	12	22	9	45		
16	9	16	12	13	19	13	19	10	36		
18	7	18	9			14	17				
20	6	20	8								
21	5										

Eppendorf microfuge 3200 (12,000 rpm) C 1.5-ml tube = 8.0
C 0.5-ml tube = 7.6

Clearing time in hours = 100 C/S, where S = sedimentation coefficient and C = K/100

$$K = \frac{1n \frac{Rmax}{Rmin}}{\omega^2} \times \frac{10^{13}}{3600}$$

$$K = \frac{2.5 \times 10^5 \; 1n \frac{Rmax}{Rmin}}{(K\ rpm)^2}$$

λ DNA	S ≅ 34
λ phage	S ≅ 400
E. coli cell	S ≅ 10^5

JA-21 ≅ SE-12
JA-20 ≅ SS-34
JA-14 ≅ GSA
JS-13 ≅ HB-4
JA-10 ≅ GS-3
JS-7.5 ≅ HS-4

IV. Units

1 μg = 1 γ = 10^{-6} g

1 ng = 10^{-9} g

1 kb = 1 kilobase of single-stranded nucleic acid

1 kb = 1 kilobase pair of duplex nucleic acid

1 kb = 6.6×10^5 daltons, duplex DNA (sodium salt)

1 kb = 3.3×10^5 daltons, single-stranded DNA (sodium salt)

1 kb = 3.4×10^5 daltons, single-stranded RNA (sodium salt)

1 kb DNA = 333-amino-acid coding capacity ≅ 37,000 daltons

10,000-dalton protein = 270 bp DNA

30,000-dalton protein = 810 bp DNA

50,000-dalton protein = 1.35 kb DNA

100,000-dalton protein = 2.7 kb DNA

200,000-dalton protein = 5.4 kb DNA

1 μg/ml of nucleic acid = 3.0 μM phosphate

1 μg/ml of a 1 kb nucleic acid = 3 nM end concentration

1 O.D. duplex nucleic acid = A_{260} of 1.0 ≅ 50 μg/ml

1 O.D. single-stranded nucleic acid = A_{260} of 1.0 ≅ 40 μg/ml

APPENDIX 7

RESTRICTION ENDONUCLEASE CLEAVAGE

I. Method

1. Add 18 µl (minus volume of DNA) H_2O to a polypropylene microfuge tube.

2. Add 2 µl 10× restriction buffer (either high-, medium-, or low-salt buffer; see II [below] for appropriate condition).

3. Add appropriate amount of DNA in 10 mM Tris (pH 7.4) and 1 mM Na_2 EDTA.

4. Add restriction enzyme. In general, 1 unit of enzyme cleaves 1 µg of DNA in 15 minutes. Mix well.

5. Incubate according to temperature in II (below) (generally at 37°C) for 30 minutes.

6. Heat to 70°C for 5 minutes to inactivate restriction enzyme and contaminating enzymes, and to melt λ cohesive ends.

II. Properties of Restriction Endonucleases

Enzyme	Salt	Temp (°C)	Activity after 70°C, 5 min	Sequence	Sites λ	Sites pBR322
AccI	med	37		GT'($^{AG}_{CT}$)AC	7	2
AluI	med	37		AG'CT	>50	16
AsuI				G'GNCC	>30	15
AvaI	med	37		G'PyCGPuG	8	1
AvaII	med	37		G'G($^{A}_{T}$)CC	>17	8
AvrII	low	37		CCTAGG	2	0
BalI				TGG'CCA	15	1
BamHI	med	37	+	G'GATCC	5	1
BbvI	low	37		GC($^{T}_{A}$)GC	>30	21
BclI	med	60	+	T'GATCA	7	0
BglI	med	37		GCCNNNN'NGGC	22	3
BglII	med	37		A'GATCT	5	0
BpaI				GT'($^{C}_{A}$)($^{G}_{T}$)AC	7	1
BpuI					6	2
BstEII	med	60	+	G'GTNACC	11	0
BstNI	low	60	+	CC'($^{A}_{T}$)GG	>35	6
ClaI				AT'CGAT	12	1
DdeI	med	37		C'TNAG	>50	8
EcoRI	high	37	-	G'$\overset{*}{A}$ATTC	5	1
EcoRII	high	37		'CC($^{A}_{T}$)GG	>35	6
Fnu4HI	low	37		GC'NGC	>50	42
FnuDII	low	37		CG'CG	>50	23
HaeI	low			($^{A}_{T}$)GG'CC($^{T}_{A}$)	-	7
HaeII	low	37		PuGCGC'Py	>30	11
HaeIII	low	37		GG'$\overset{*}{C}$C	>50	22
HgaI	med	37		GACGCNNNNN' CTGCGNNNNNNNNNN'	>50	11
HgiAI	high	37		G($^{T}_{A}$)GC($^{T}_{A}$)'C	20	8
HhaI	med	37		GCG'C	>50	31
HincII	med	37		GTPy'PuAC	34	2
HindII	med	37		GTPy'PuAC	34	2
HindIII	med	37	+	A'AGCTT	6	1

Enzyme	Salt	Temp (°C)	Activity after 70°C, 5 min	Sequence	Sites λ	Sites pBR322
HinfI	med	37		G'ANTC	>50	10
HpaI	low	37		GTT'AAC	11	0
HpaII	low	37		C'$\overset{*}{C}$GG	>50	26
HphI	low	37		GGTGANNNNNNNN' CCACTNNNNNNN'	>50	12
KpnI	low	37	-	GGTAC'C	2	0
MboI	high			'GATC	>50	22
MboII	low	37		GAAGANNNNNNNN' CTTCTNNNNNNN'	>50	11
MnlI	high	37		CCTC	>50	26
MspI	low	37		C'CGG	>50	26
MstI				TGCGCA	>10	4
PstI	med	30	-	CTGCA'G	18	1
PvuI	high	37		CGATCG	3	1
PvuII	med	37		CAG'CTG	15	1
RsaI	med	37		GT'AC	>50	3
SacI	low	37		GAGCT'C	2	0
SacII	low	37		CCGC'GG	4	0
SacIII	high			ACGT	>10	6
SalI	high	37	+	G'TCGAC	2	1
Sau3AI	med	37		'GATC	>50	22
Sau96I	med	37		G'GNCC	>30	15
SmaI	(1)	37		CCC'GGG	3	0
SstI	low	37		GAGCT'C	2	0
SstII	low	37		CCGC'GG	3	0
SstIII	high			ACGT	>10	6
TaqI	low	65	+	T'CGA	>50	7
ThaI	low	60	+	CG'CG	>50	23
XbaI	high	37		T'CTAGA	1	0
XhoI	high	37	-	C'TCGAG	1	0
XmaI	low	37		C'CCGGG	3	0

Buffers	NaCl	Tris	$MgSO_4$	DTT
Low	0	10 mM (pH 7.4)	10 mM	1 mM
Med	50 mM	10 mM (pH 7.4)	10 mM	1 mM
High	100 mM	50 mM (pH 7.4)	10 mM	0
(1)	20 mM KCl	10 mM (pH 8)	10 mM	1 mM

APPENDIX 8

λ VECTOR CAPACITY

		Insert size yielding normal length phage (kb)	Insert size yielding 5% overpackaged phage (kb)	Minimum insert yielding viable phage (kb)	Relevant genetic markers		
					att	red	imm
EcoRI	λgt1-λB	13.4	15.9	1.1	-	-	λcI857
EcoRI	λgt4-0	10.0	12.5	0	+	+	λcI857
EcoRI	λgt5-lac5	15.6	18.0	3.4	-	-	λcI857
EcoRI	λsep6-lac5^2	18.0	20.5	5.8	-	-	21cI$^-$
EcoRI	λgt7-ara6	14.2	16.6	1.5	-	+	λcI$^-$
EcoRI	Charon 4	18.8	21.3	6.6	-	-	λcI$^-$
EcoRI	λ607	9.2	11.6	0	-	+	434cI^{+*}
HindIII	λ590	9.2	11.6	0	-	+	434cI^{+*}
HindIII	λ760	14.7	17.1	2.4	-	-	λcI$^-$
SalI	λgt30-Ec6	16.4	18.9	4.2	-	+	21cI$^-$
SstI	λgt40-0	9.7	12.1	0	-	-	λcI857

* Insertions are in the 434cI gene; therefore, hybrids are cI$^-$ and give clear plaques.

APPENDIX 9

RESTRICTION MAPS

I. λ Maps and λ Vectors

Some of the λ vectors that have been used to clone foreign DNA and are useful vectors are described in the preceding pages. Many λ vectors have been developed, but most of these have not been tested as vectors by cloning foreign DNA, or they have not been optimally designed.

Figure 1 shows the λ map with the λ coordinates (fractional length positions on λ+) of a number of the λ genes. Below this map are a number of deletions and substitutions. Above the name of the region is the net size change in kilobase pairs (kb) of λ DNA having the deletion or substitution (i.e., immunity region i21 is 2.45 kb shorter than λ+). Below the line representing the substitution is the size of non-λ sequences (in kb). The λ coordinates are given at the ends of each deletion or substitution.

Figures 2 and 3 show restriction endonuclease cleavage sites in λ DNA. The λ coordinates were determined by P. Philippsen and R.W. Davis (in prep.) using electron microscopy and agarose gel electrophoresis comparing lengths and mobilities to those of ϕX174. There is excellent agreement between electron microscopy and gel electrophoresis except for fragments near coordinate 0.85. Fragments in this region were slightly longer when measured by electron microscopy as compared with relative mobility measurements. The map is an average of the two sets of determinations. Also, data from Daniels et al. (1980) are scaled and added to the above maps. Their work does not disagree significantly with our determinations. λ^+ is determined to be 49.0 kb. The numbers above the bars are the sizes (in kb) of the corresponding fragments. The numbers below the bars are fractional length positions of λ^+.

Figures 4 through 14 show λ vector DNAs as cross-hatched. Deletions are drawn as thin lines with the deletion name above the

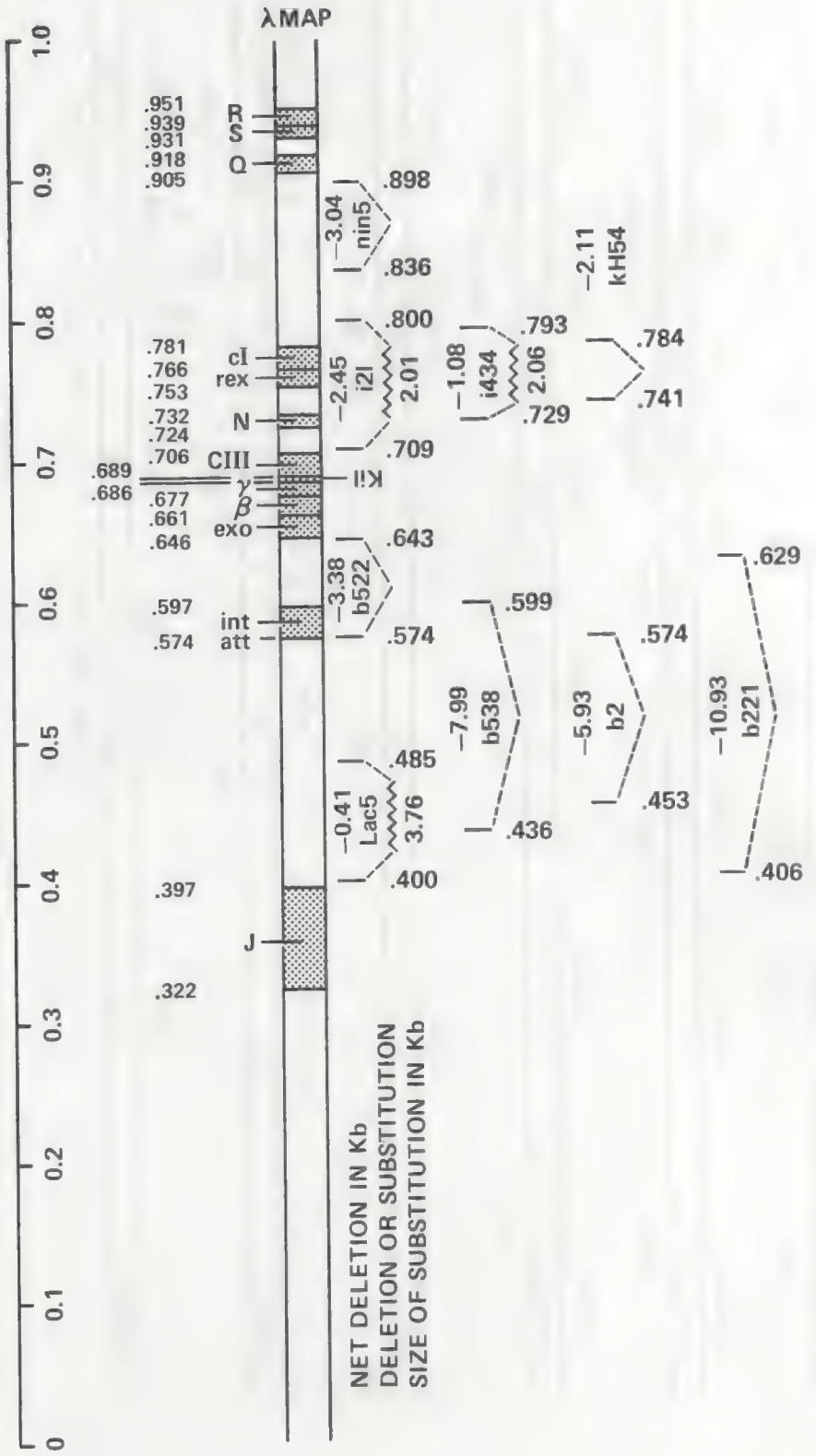
line. Non-λ sequences are heavily dotted areas slightly above the line (i.e., the 21 immunity region in Fig. 9 or ϕ80 DNA in Fig. 10). Also, ϕ80 DNA is shown in Figures 11 and 12, on the right arm of λ that removes the HindIII site. Sequences that are present in the vector but are usually discarded in hybrids (replacement vectors) are shown as lightly dotted areas above the λ bars. The λ coordinates for each disruption in the λ sequence are shown below the λ bars. Just above the λ bars, the size of each DNA segment is given in kb. The total length of the λ left arm and right arm of hybrids is given in kb.

Reference

Daniels, D.L., J.R. de Wet, and F.R. Blattner. 1980. J. Virol. (in press).

See Sanger et al JMB 162:729-773 (1982) — Complete DNA seq. of λ — 48,502 bp

FIGURE 1


λMAP
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
.951
.939
.931
.918
.905
R
S
Q
.898
−3.04
nin5
.836
−2.11
kH54
.800
.793
.784
.781
.766
.753
cI
rex
−2.45
i2I
2.01
−1.08
i434
2.06
.741
.729
.732
.724
.706
N
CIII
.709
.689
.686
γ
K II
.677
.661
.646
β
exo
.643
−3.38
b522
.597
.574
int
att
.574
.599
−7.99
b538
.574
−5.93
b2
.629
−10.93
b221
.485
−0.41
Lac5
3.76
.436
.453
.406
.400
.397
J
.322
NET DELETION IN Kb
DELETION OR SUBSTITUTION
SIZE OF SUBSTITUTION IN Kb

1983 – Can get exactly fr. Sanger sequences –

CLEAVAGE SITES FOR RESTRICTION ENDONUCLEASES IN λ DNA

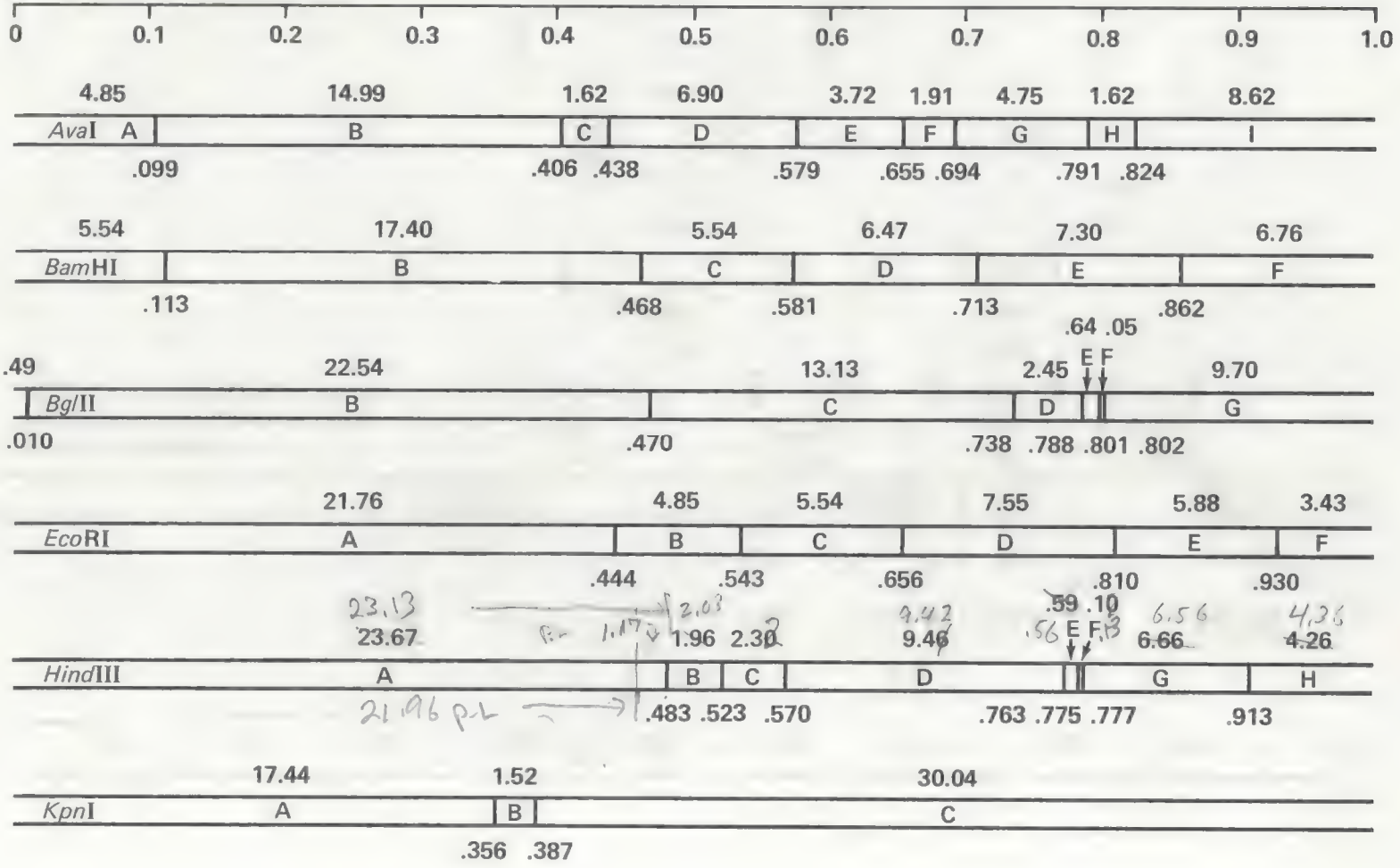


FIGURE 2

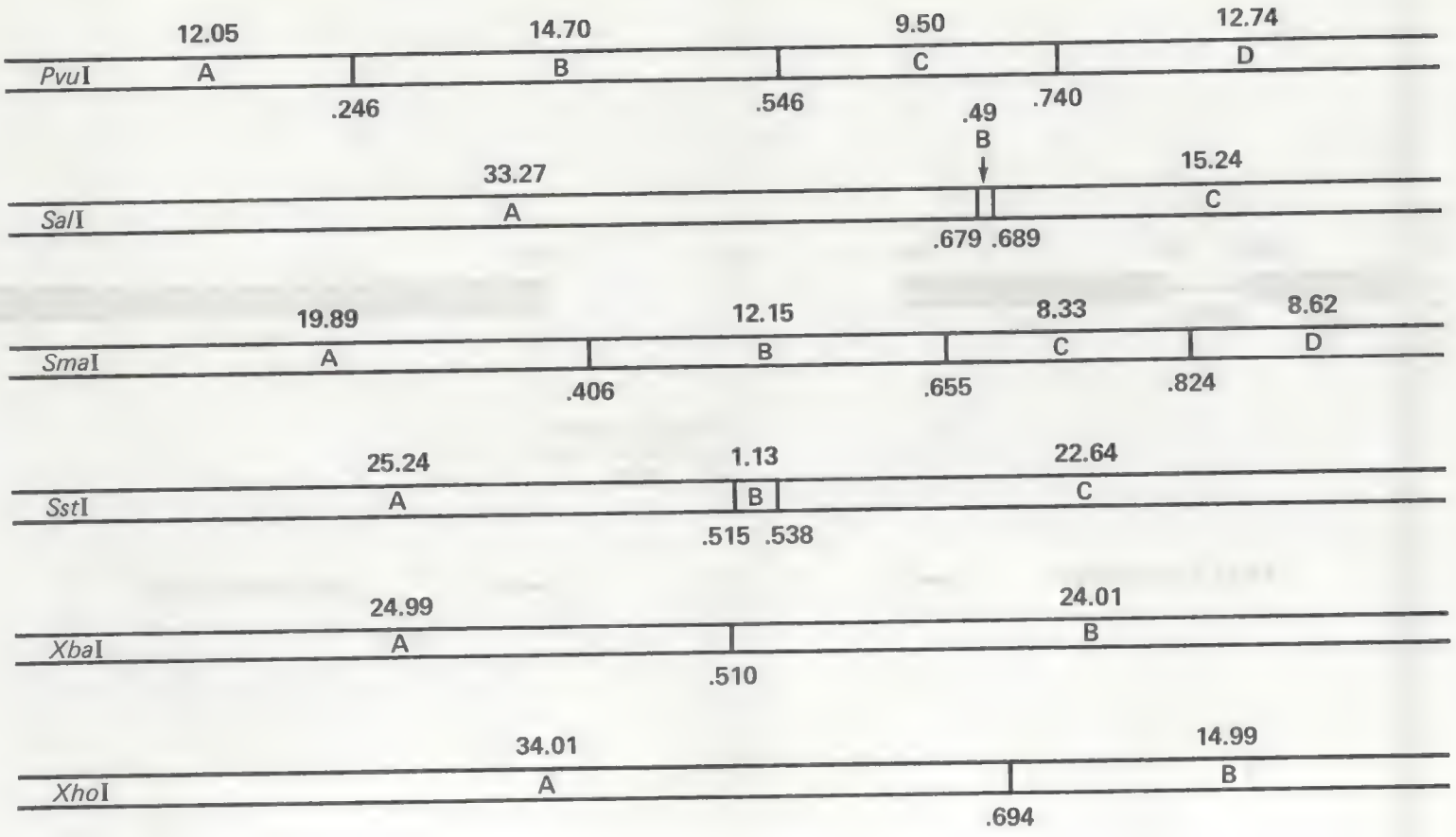

PvuI
12.05
A
14.70
B
9.50
C
12.74
D
.246
.546
.740
SalI
33.27
A
.49
B
15.24
C
.679
.689
SmaI
19.89
A
12.15
B
8.33
C
8.62
D
.406
.655
.824
SstI
25.24
A
1.13
B
22.64
C
.515
.538
XbaI
24.99
A
24.01
B
.510
XhoI
34.01
A
14.99
B
.694

FIGURE 3

FIGURE 4

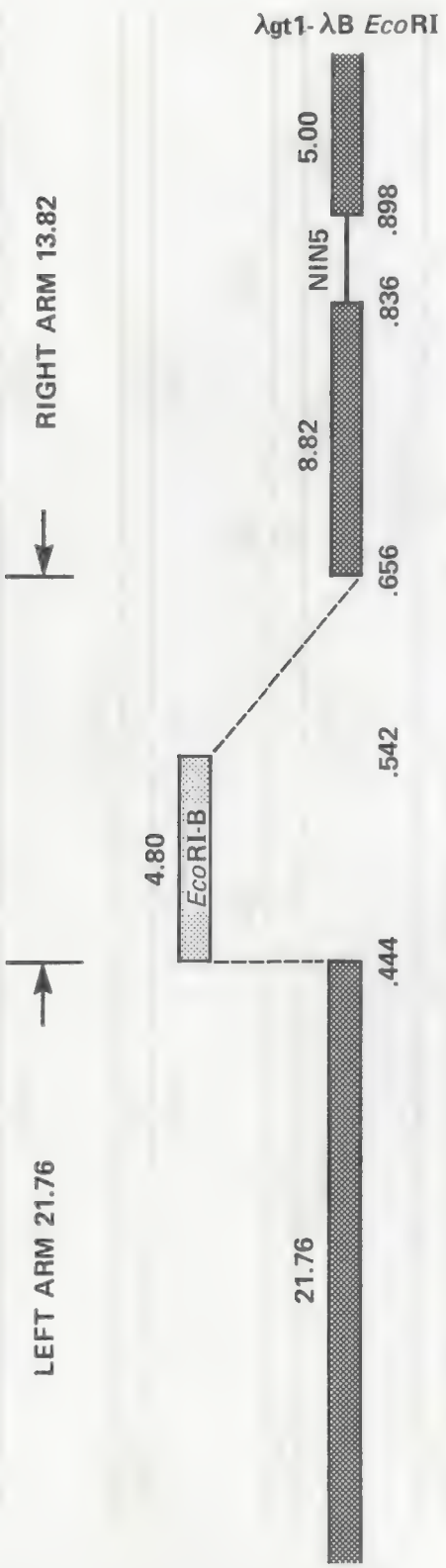

λgt1-λB EcoRI
RIGHT ARM 13.82
LEFT ARM 21.76
5.00
.898
NIN5
.836
8.82
.656
.542
4.80
EcoRI-B
.444
21.76

FIGURE 5

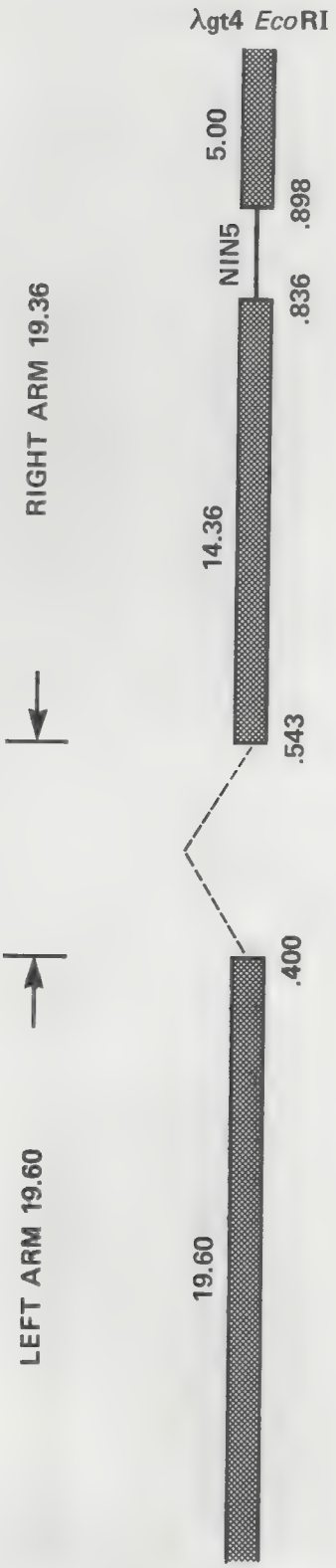

λgt4 EcoRI
5.00
.898
NIN5
.836
14.36
.543
.400
19.60
RIGHT ARM 19.36
LEFT ARM 19.60

FIGURE 6


λgt5 – lac5 EcoRI
5.0
.898
NIN5
.836
8.82
.656
RIGHT ARM 13.82
6.60
LAC5
.400
19.60
LEFT ARM 19.60

FIGURE 7

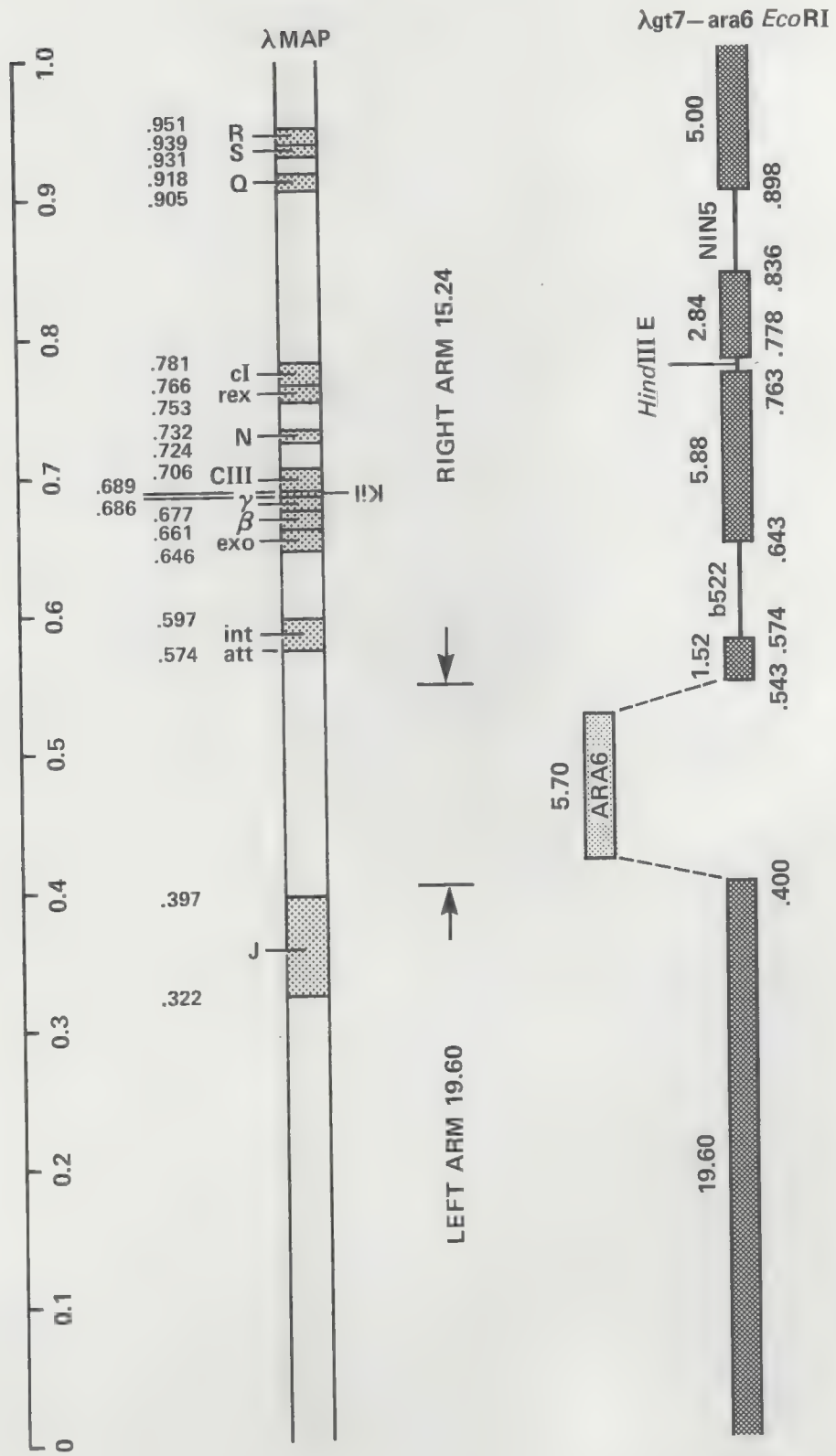

λMAP
λgt7–ara6 EcoRI
RIGHT ARM 15.24
LEFT ARM 19.60
HindIII E
ARA6
NIN5
b522
R
S
Q
cI
rex
N
CIII
γ
β
exo
int
att
J
kil
5.00
2.84
5.88
1.52
5.70
19.60
.951
.939
.931
.918
.905
.781
.766
.753
.732
.724
.706
.689
.686
.677
.661
.646
.597
.574
.397
.322
.898
.836
.778
.763
.643
.574
.543
.400
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

FIGURE 8

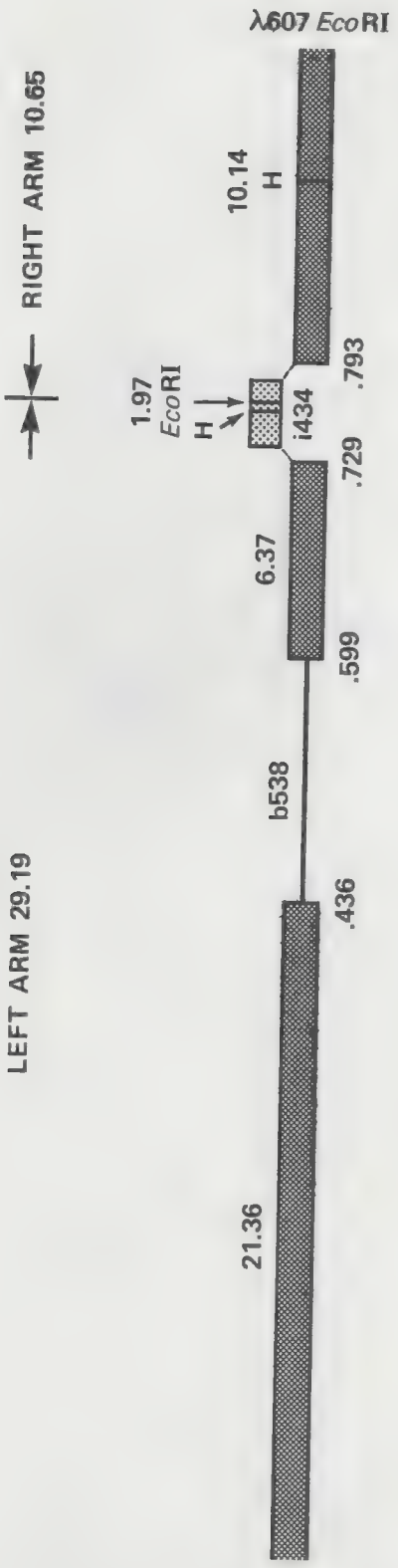

λ607 EcoRI
RIGHT ARM 10.65
10.14
H
1.97
EcoRI
H
i434
.793
.729
6.37
.599
b538
.436
LEFT ARM 29.19
21.36

FIGURE 9

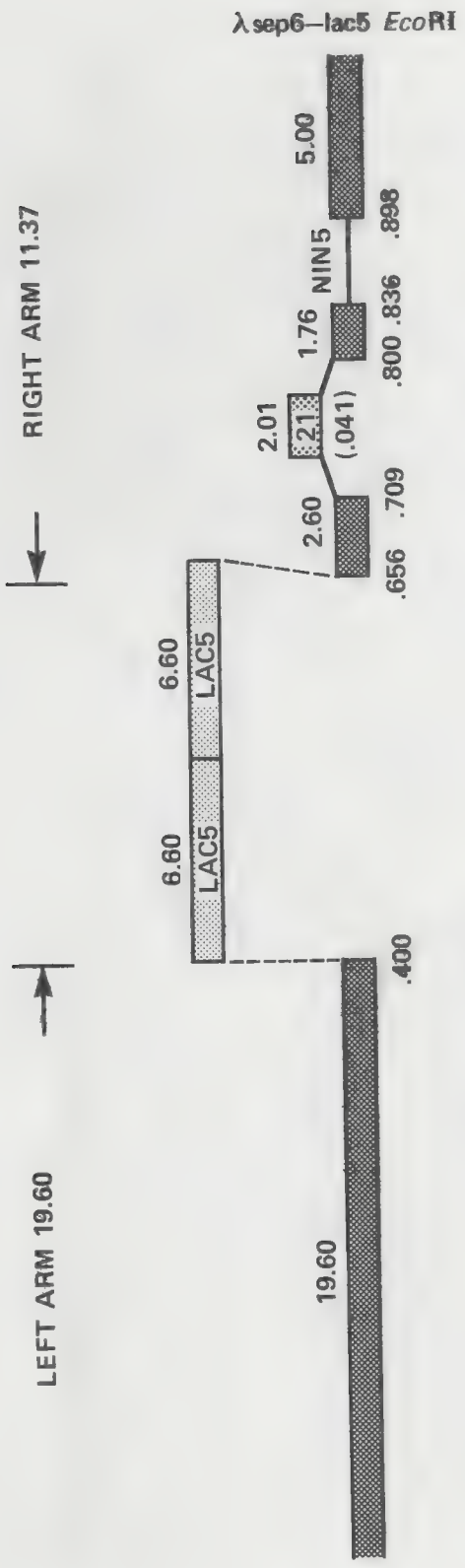

λsep6–lac5 EcoRI
5.00
.898
NIN5
.836
1.76
.800
2.01
21
(.041)
2.60
.709
.656
RIGHT ARM 11.37
6.60
LAC5
6.60
LAC5
.400
19.60
LEFT ARM 19.60

FIGURE 10

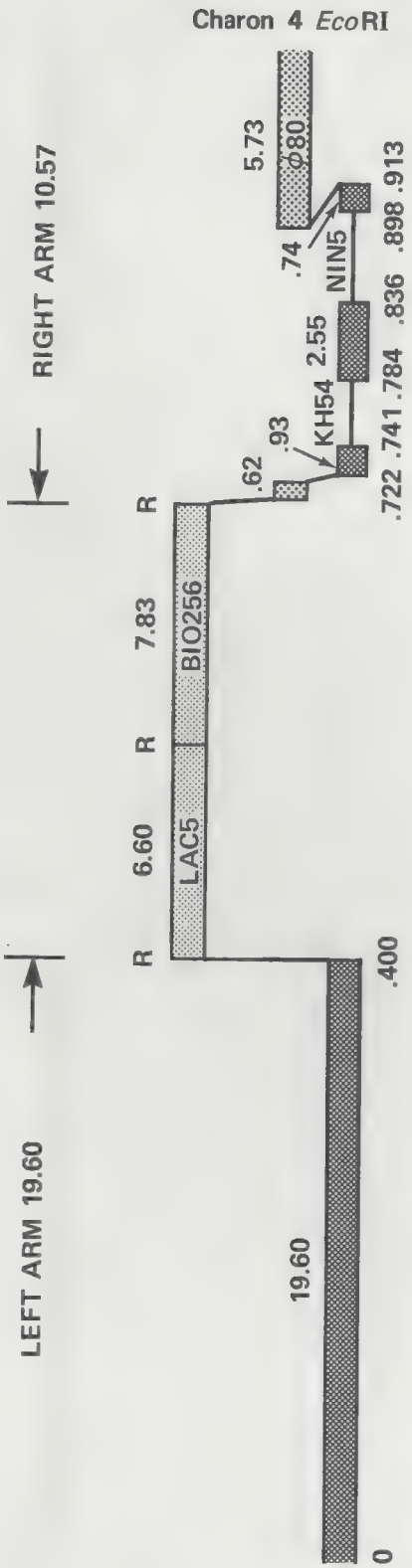

Charon 4 EcoRI
RIGHT ARM 10.57
LEFT ARM 19.60
5.73
Φ80
.74
NIN5
.898 .913
.836
2.55
KH54
.784
.741
.722
.93
.62
R
7.83
BIO256
R
6.60
LAC5
R
.400
19.60
0

FIGURE 11

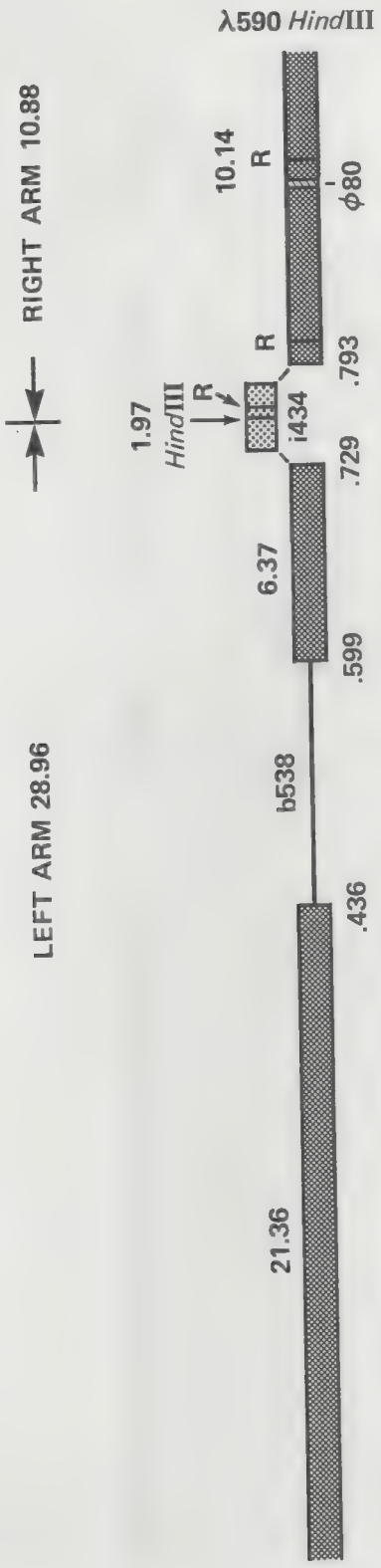

λ590 HindIII
RIGHT ARM 10.88
LEFT ARM 28.96
10.14
R
φ80
R
.793
1.97
HindIII
R
i434
.729
6.37
.599
b538
.436
21.36

FIGURE 12

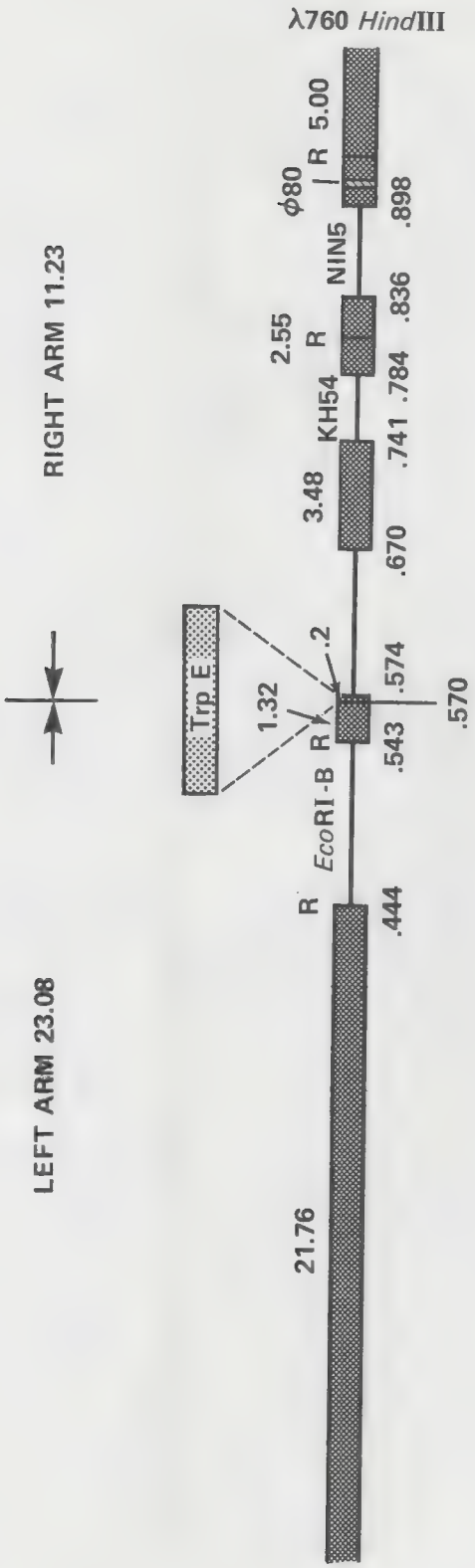

λ760 HindIII
RIGHT ARM 11.23
LEFT ARM 23.08
5.00
R
φ80
.898
NIN5
.836
2.55
R
.784
KH54
.741
3.48
.670
.574
.2
1.32
Trp E
R
.543
.570
EcoRI-B
R
.444
21.76

FIGURE 13

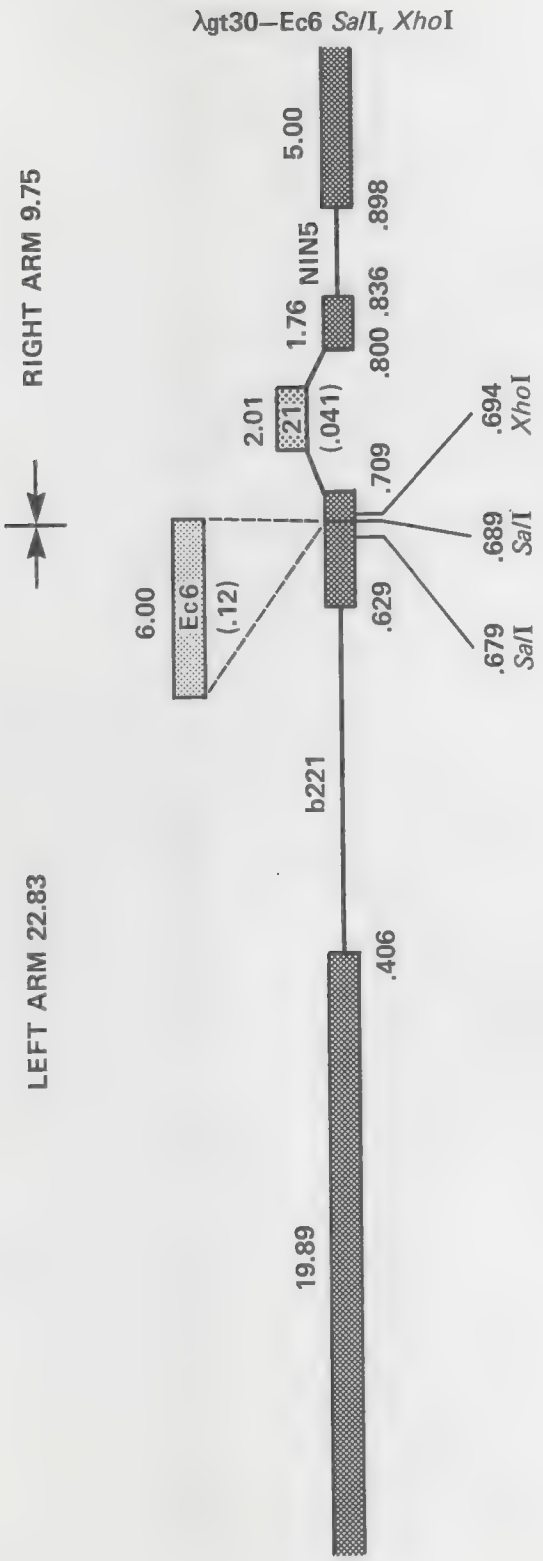
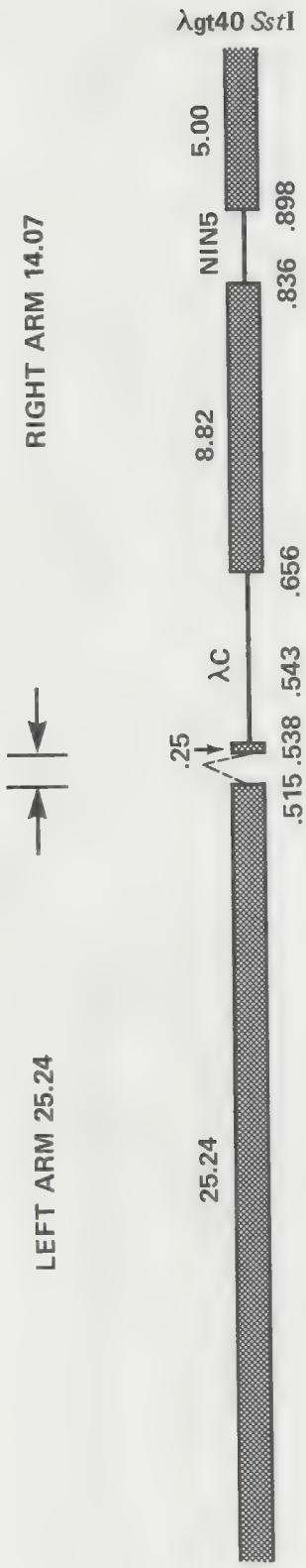

λgt30–Ec6 SalI, XhoI
RIGHT ARM 9.75
LEFT ARM 22.83
5.00
.898
NIN5
.836
1.76
.800
2.01
221
(.041)
.709
.694
XhoI
.689
SalI
.679
SalI
.629
6.00
Ec6
(.12)
b221
.406
19.89

FIGURE 14

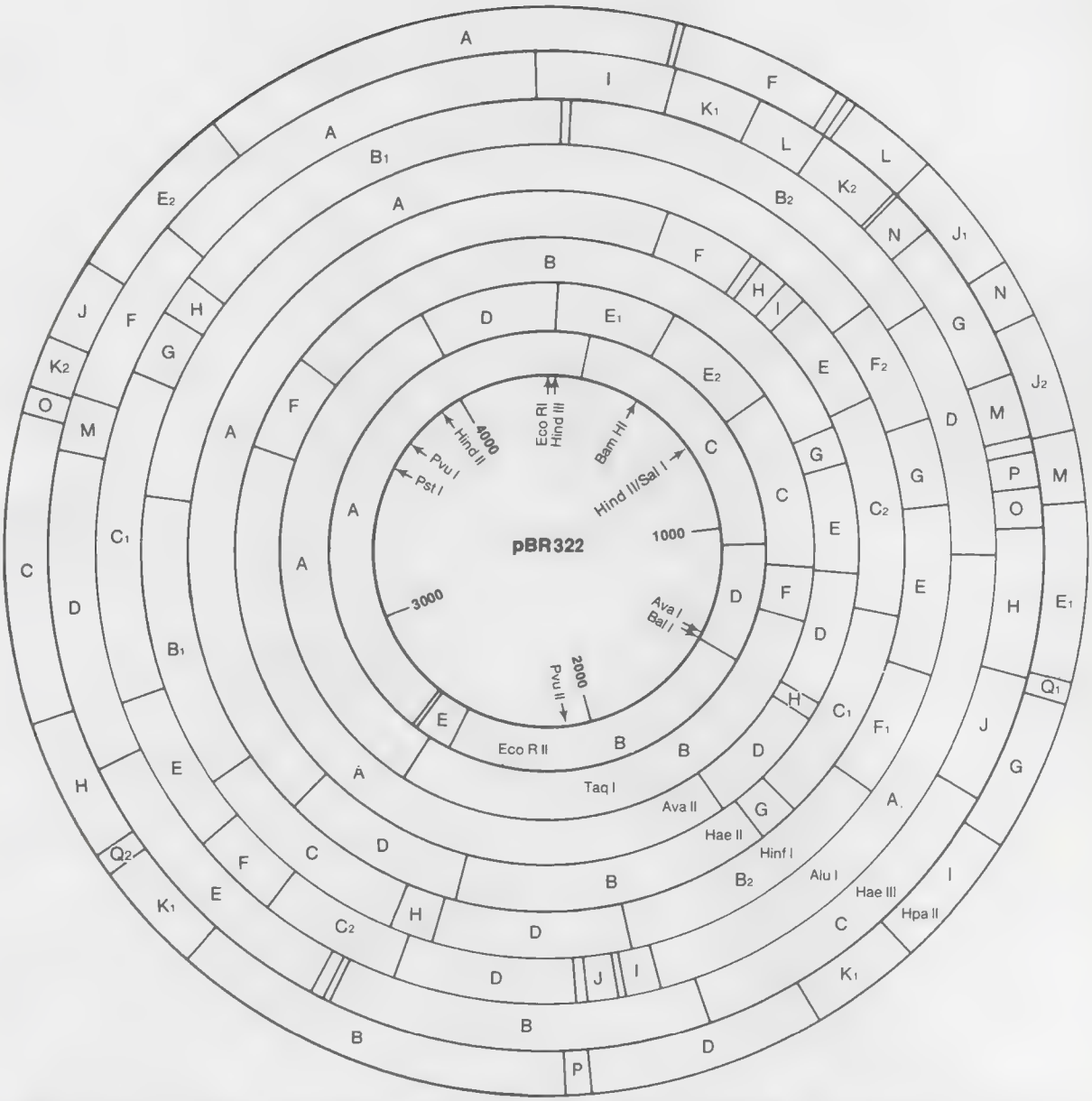

λgt40 SstI
5.00
.898
NIN5
.836
8.82
.656
λC
.543
.538
.25
.515
25.24
RIGHT ARM 14.07
LEFT ARM 25.24

II. Map of pBR322 2.6 Mol

The plasmid pBR322 is the most popular plasmid in general use for subcloning fragments in E. coli. It carries an origin of replication derived from Colicin E1 which will replicate itself and any DNA attached to it to a level of about 30 copies per cell. pBR322 also carries genes conferring upon cells that carry it resistance to high levels of the antibiotics tetracycline and ampicillin. The Tet^R gene lies in the region of nucleotides 0–1500 on the physical map in Figure 15 and the Amp^R gene lies in the region of nucleotides 3600–4300. The origin of replication lies in the region around nucleotide 2500.

There are a number of restriction targets that occur only once in pBR322 and that therefore can be used for cloning. These include BamHI, SalI, and HindIII (which lie within the Tet^R gene), PvuI and PstI (which lie within the Amp^R gene), and EcoRI, AvaI, PvuII, ClaI, and BalI (which lie within no sequence known to be important for drug resistance or replication).

FIGURE 15



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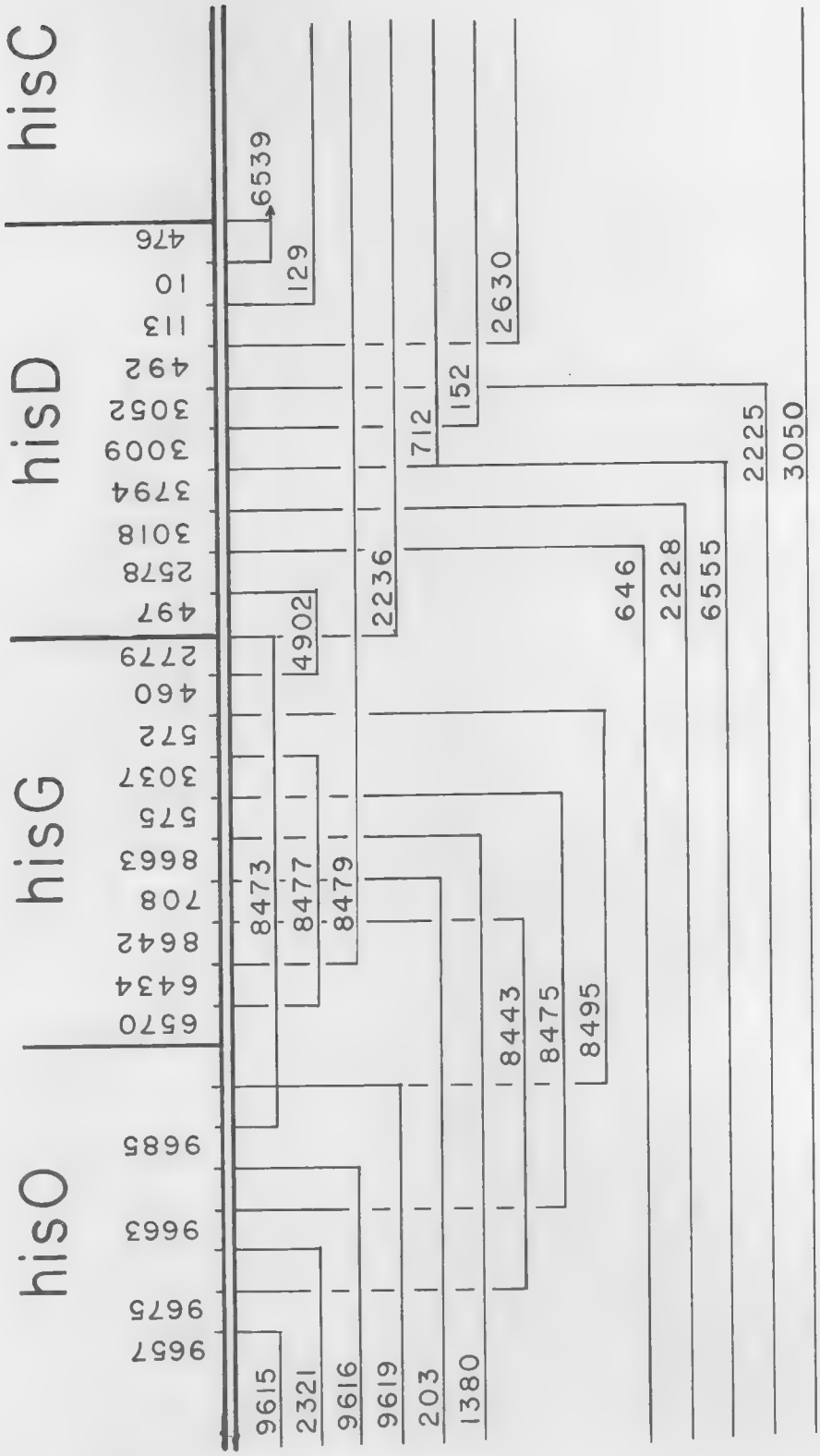
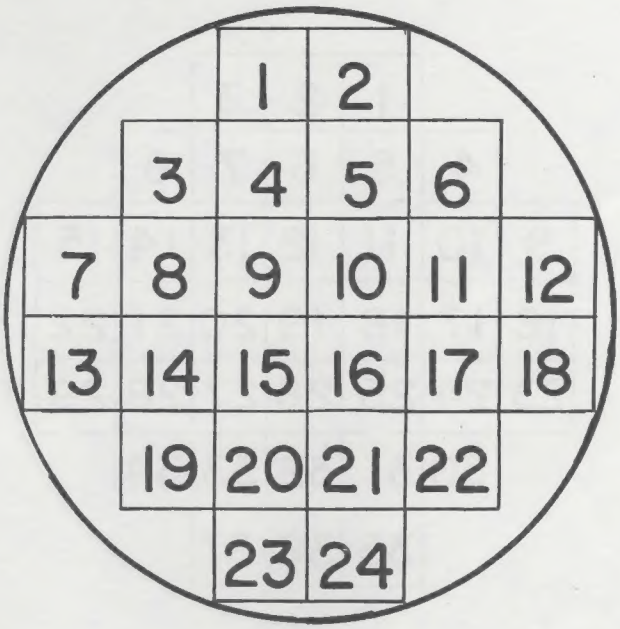
APPENDIX 10

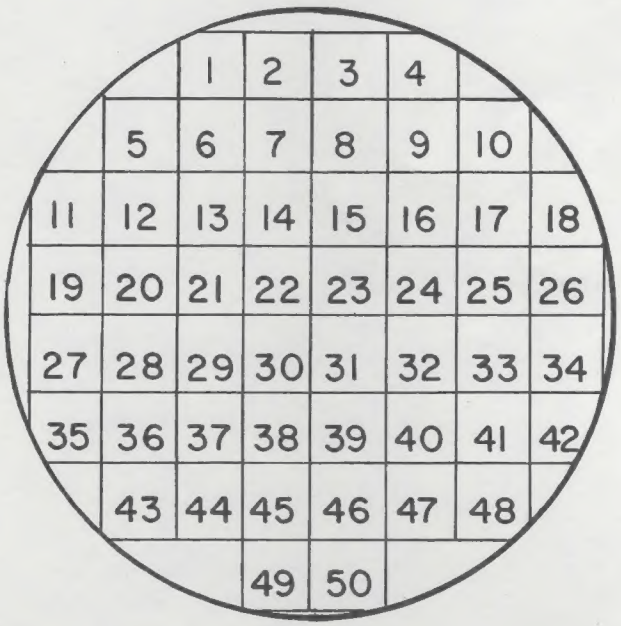
HISTIDINE DELETION MAP

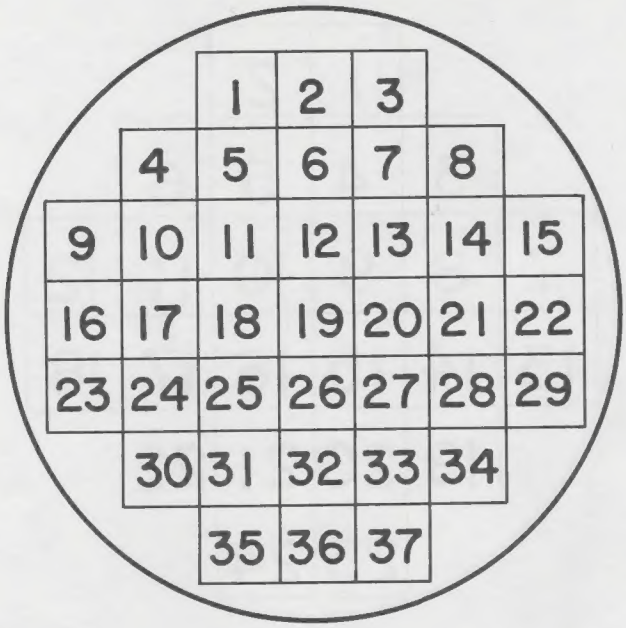


APPENDIX 11

PATCH PATTERN






1 2 3
4 5 6 7 8
9 10 11 12 13 14 15
16 17 18 19 20 21 22
23 24 25 26 27 28 29
30 31 32 33 34
35 36 37

Davenport Laboratory, built in 1927, has been the site of genetics courses at Cold Spring Harbor Laboratory for more than thirty-five years. The first such course, on bacterial viruses, was taught in this building by Max Delbrück in 1945. Among the many eminent scientists who have participated in the courses are Seymour Benzer, Herman Kalckar, Aaron Novick, Leo Szilard, and Gunther Stent.

The original building (shown on the front cover as it appeared in the early 1950s) was designed by Henry H. Saylor, a Huntington, Long Island, architect. Construction of an extension, designed by the firm of Moore Grover Harper of Essex, Connecticut, was begun in 1980. Reflecting both the past history and the future promise of genetics research, the combined structure (depicted in the architect's model below) will be named Delbrück Laboratory.

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